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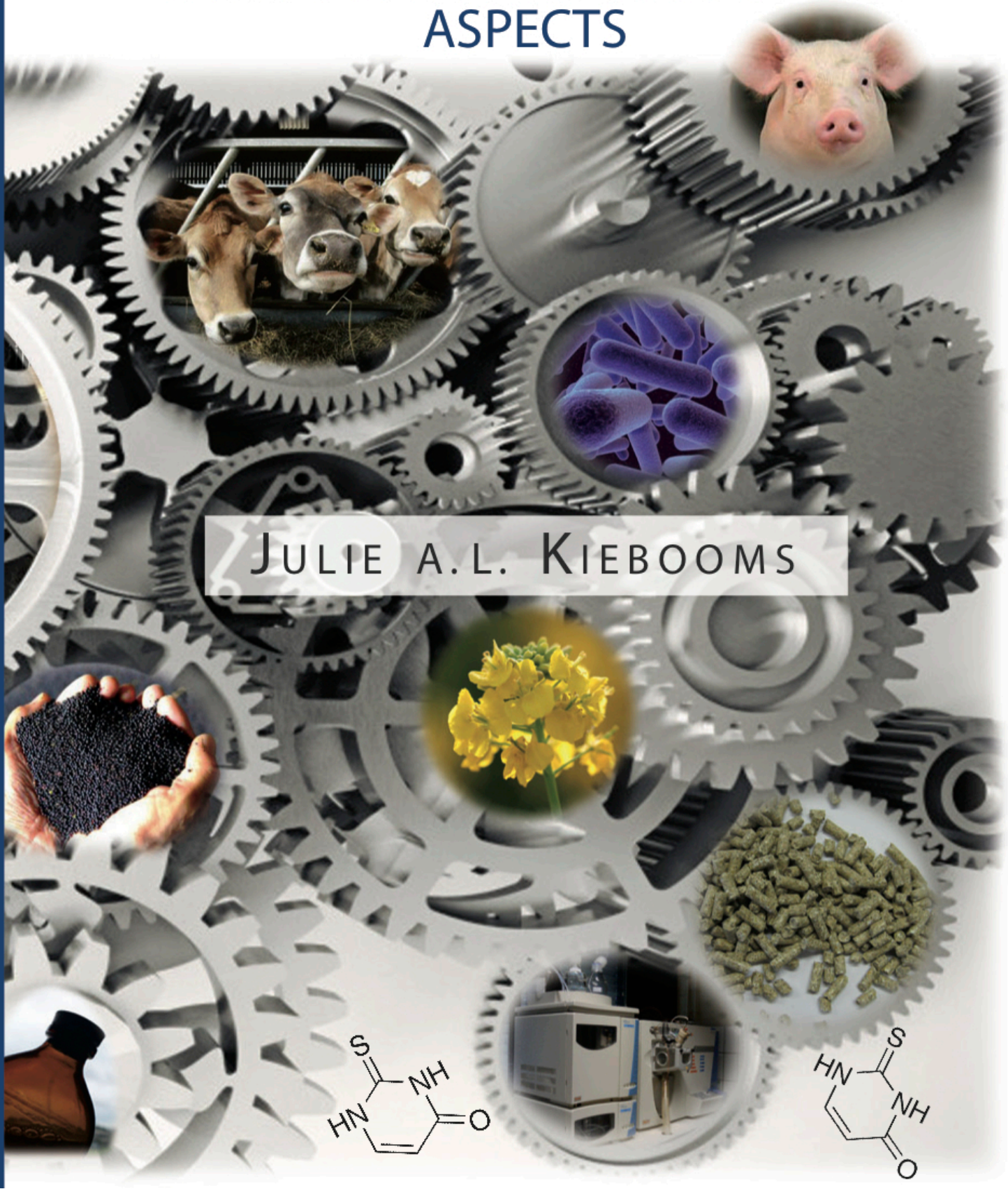
ENDOGENOUS FORMATION OF THIOURACIL IN LIVESTOCK: MECHANISTIC AND ANALYTICAL ASPECTS



JULIE A.L. KIEBOOMS

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Endogenous formation of thiouracil in livestock: mechanistic and analytical aspects

Julie A. L. Kiebooms

Thesis submitted in the fulfilment of the requirements for the degree of Doctor in Veterinary Sciences (PhD), Faculty of Veterinary Medicine, Ghent University

Promoter:
Prof. dr. ir. Lynn Vanhaecke

Co-Promoter:
Dr. Julie Vanden Bussche

Ghent University
Faculty of Veterinary Medicine
Department of Veterinary Public Health and Food Safety
Laboratory of Chemical Analysis

Promoters

Prof. dr. ir. L. Vanhaecke
UGent, Fac. of Veterinary Medicine, Dept. Veterinary Public Health and Food Safety

Dr. J. Vanden Bussche
UGent, Fac. of Veterinary Medicine, Dept. Veterinary Public Health and Food Safety

Members of the reading committee

Prof. dr. S. Croubels
UGent, Fac. of Veterinary Medicine, Dept. Pharmacology, Toxicology and Biochemistry

Prof. dr. S. Daminet
UGent, Fac. of Veterinary Medicine, Dept. of Medicine and Clinical Biology of Small Animals

Prof. dr. B. Le Bizec
ONIRIS, Laboratoire d'étude des Résidus et Contaminants dans les Aliments

Members of the examination committee

Prof. dr. Em. H. F. De Brabander,
Representative of the dean

Prof. dr. K. Houf
UGent, Fac. of Veterinary Medicine, Dept. Veterinary Public Health and Food Safety

Prof. dr. ir. V. Fievez
UGent, Fac. of Bioscience Engineering, Dept. Animal Production

Dr. S. Sterk
RIKILT-Institute for Food Safety, European Reference Laboratory

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a.u.	Arbitrary units
AA	Area under the peak
ACN	Acetonitrile
AFLP	Amplified fragment length polymorphism
ANF	Anti-nutritional factors
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photoionisation
BTU	Benzyl-6-thiouracil
CANOLA	CAnadian Oil, Low Acid
Cc_α	Decision limit
Cc_β	Detection limit
CCF	Central composite face design
CCM	Coarse colza '00' meal
CE	Capillary electrophoresis (CE)
CEIA	Chemiluminescent-enzyme-immunoassay
CIA	Chemiluminescent immunoassay
CID	Collision induced dissociation
ClO_4^-	Perchlorate
Da	Dalton or atomic mass unit (u)
DAD	Diode-array detection
DC	Direct current
DIT	Di-iodinated tyrosine
DL	Direct liquid injection
DMTU	6-dimethyl-thiouracil
DNA	Deoxyribonucleic acid
EC	Enzyme commission number (cfr. myrosinase)
EC	European Commission (cfr. legislation)
ED	Equilibrium dialysis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light scattering detection
EtOH	Ethanol
ETU	6-ethyl-thiouracil
EU	European Union
EURL	European Union Reference Laboratories
FAFLP	Fluorescent AFLP
FWHM	Full width half maximum

GC	Gas chromatography
GI	Gastro-intestinal
GL(s)	Glucosinolate(s)
h	Hours
HCl	Hydrochloric acid
(H)ESI	(Heated) electrospray ionisation
HRMS	High resolution mass spectrometry
3-IBBr	3-Iodobenzyl bromide
i-SCN^-	Isothiocyanate
ID	Isotope dilution
IFN-γ	Interferon-gamma
IP-HFLPM	Ion-pair based hollow fiber liquid phase microextraction
LC	Liquid chromatography
LC-MS^2	Liquid chromatography multiple mass spectrometry
LIF	Laser-induced fluorescence (LIF)
LOD	Limit of detection
LOF	Lack of fit test
LOQ	Limit of quantification
m/z	Mass-to-charge-ratio
MBI	2-mercaptobenzimidazole
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
Min	Minutes
MIT	Mono-iodinated tyrosine
MLST	Multilocus sequence typing (MLST)
MRPL	Minimum required performance limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	N-methyl-B-trimethylsilyl-trifluoroacetamide
n.a.	Not available
n.d.	Not detected
NaCl	Sodium chloride
NO_3^-	Nitrate
OF	Oxidative fermentative test
OZT(s)	Oxazolidine-2-thione(s)
PB	Particle beam
PB	Plackett-Burman (cfr. statistical design)
PCR	Polymerase chain reaction

PFBBr	Pentafluorobenzylbromide
PFGE	Pulse-field gel electrophoresis
PhTU	6-phenyl-thiouracil
PTU	6-propyl-2-thiouracil
PTU-d5	6-propyl-d_5-2-thiouracil
Q	Quadrupole
QqQ	Triple quadrupole mass analyser
R	Variable side chain
R^2	Determination coefficient
RAPD	Random amplified polymorphic DNA
RC	Recommended concentration
(r)DNA	(ribosomal) Deoxyribonucleic acid
REM	Rapeseed extraction meal
RF	Radiofrequency
RFLP	Restriction fragment length polymorphism
RIA	Radio immunoassay
(r)RNA	(ribosomal) Ribonucleic acid
RS	Rapeseed
RSD%	Percentual relative standard deviation or (CV%) coefficient of variation
RSM	Response surface modelling
rT_3	Reverse triiodothyronine or 3,3',5'-triiodothyronine
SCFA(s)	Short chain fatty acid(s)
SCN^-	Thiocyanate
SD	Standard deviation
SN	Signal-to-noise ratio
SPE	Solid phase extraction
SRM	Selected reaction monitoring
T_3	Triiodothyronine or 3,3',5-triiodothyronine
T_4	Thyroxin or 3,5,3',5'-tetraiodothyronine
TAP	1-methyl-2-mercaptoimidazole or tapazole
Tg	Thyroglobulin
TGB	Thyroxin binding globulin
TLC	Thin layer chromatography
ToF	Time-of-flight
TPO	Thyroid peroxidase enzyme
TRH	Thyroid releasing hormone
TS	Thermospray

TSH	Thyroid stimulating hormone
TU	4(6)-R-2-thiouracil
UF	Ultrafiltration
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet detection
5-VOT	5-vinyl-1,3-oxazolidine-2-thione

CHAPTER I

General Introduction

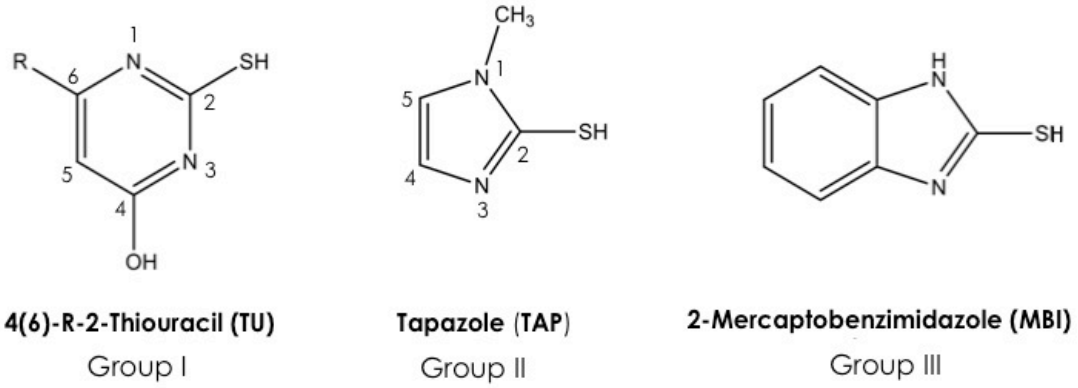
1. THYREOSTATIC DRUGS

Thyreostatic drugs are thyroid inhibiting synthetic compounds, which originally were elaborated for therapeutic use only, in humans and some domestic animals (e.g. cat, dog). Their primary application consists of constraining an overly active thyroid gland. This condition is called hyperthyroidism and causes an increase in thyroid hormones, thyroxine (T4 or 3,5,3',5'-tetraiodothyronine) and triiodothyronine (T3 or 3,5,3'-triiodothyronine), which have a broad influence on the organism's basal metabolism [1]. Subsequent life-deteriorating symptoms (e.g. anxiety, heart racing, weight loss despite an increased appetite, excessive sweating, more frequent bowl movements and fatigue) are associated with hyperthyroidism. Thyreostatic drugs influence the iodine uptake mechanism and as such block the exponential production of thyroid hormones. In general hyperthyroidism can be related to many causes, including autoimmune diseases (Graves' disease [2]), neoplastic (toxic thyroid adenoma, carcinoma) [2] and inflammatory processes (thyroiditis) [2], as well as, external factors (dietary iodine deficiency, medication [3]) and environmental factors (dioxins, pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs), bisphenol-A (BPA) and triclosan) [2,4,5]. The origins of hyperthyroidism, however, differ considerably with respect to their prevalence in humans and domestic animals.

1.1. Origin and specificities

Thyroid inhibiting compounds embody a diverse group of components belonging to various chemical entities, exerting different mechanisms of action. Collectively, they inhibit the thyroid function and as such referred to as goitrogens since they all induce a decrease of thyroid hormone production, with goitrogenic symptoms (goiter) as a result [1,6]. This effect can be caused either by synthetic thyreostatic drugs, dietary goitrogens or other chemicals with goitrogenic side effects. The least toxic and most effective synthetic thyreostatic compounds are usually used as therapeutic drugs in human (e.g. propylthiouracil) and veterinary (e.g. methimazole or carbimazole) medicine in the treatment of hyperthyroidism [1].

1.1.1.Synthetic goitrogenic therapeutics



4(6)-R-2-Thiouracil (TU) Group I

Tapazole (TAP) Group II

2-Mercaptobenzimidazole (MBI) Group III

Figure I.1: Different chemical structure groups of the anti-thyroid drugs.

The synthetic or therapeutic thyreostats comprise of 3 distinct structural groups. The first group consists of sulphated heterocyclic aromatic compounds containing two nitrogen atoms at positions 1 and 3, forming a pyrimidine structure (Figure I.1). The different analogues within this group have a variable substitution on position 6 with either an ethyl (E), dimethyl (DM), propyl (P), phenyl (Ph) or no side chain at all (= thiouracil) (Figure I.1). The second group only comprises tapazole (TAP) also known as, 1-methyl-2-mercaptoimidazole or methimazole, consisting of sulphated and methylated (Me) imidazole type structure. The third group refers to 2-mercaptobenzimidazole (MBI), an aromatic heterocyclic substance consisting of a benzene ring conjoined with a sulphated imidazole structure (Figure I.1) [6].

Physicochemical properties of these thiated nucleic acid bases, in which the oxygen atom in the nucleic acid base uracil is substituted by sulphur, arise from the heavier sulphur atom and the extensively conjugated π-electron system. This thioamide bond 'N-C-S' has been related to the thyroid-inhibiting activity [7-8]. For thiouracil this causes more then for the other uracil analogues acidic properties because the sulphur atom is a stronger Lewis base towards soft electron-pair acceptors. Furthermore, for thiouracil for example, 12 tautomeric forms (four are thio-enols, four are keto-enols, three are di-enols (keto-enol and thio-enol) and one is a keto tautomer) have been described due to significant polarizability of the conjugated π-electron system [1,9,10]. Six tautomers (Figure I.2) are commonly observed compared to the other forms [9].

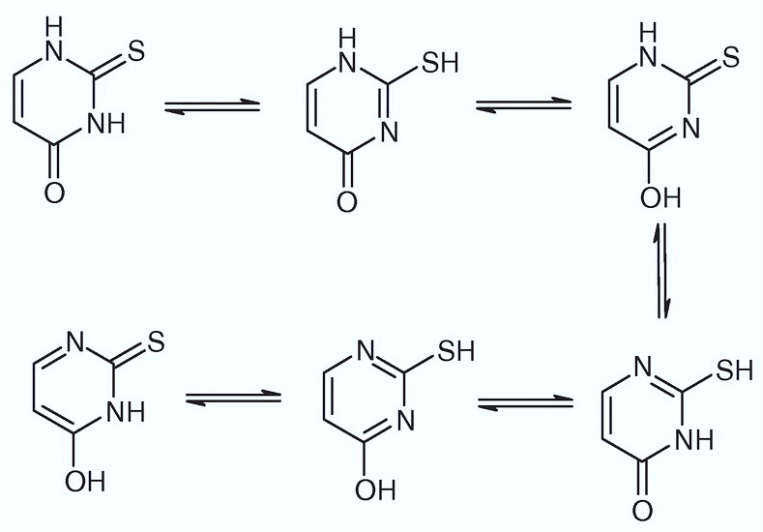


Figure I.2: The six main tautomeric forms of thiouracil [1,10].

Overall synthetic therapeutic goitrogens are very polar, amphoteric, low molecular structures with masses ranging from 114.2 Da for TAP to 204.3 Da for PhTU [1,11].

Certain of these synthetic thyreostats have been widely used as part of therapeutic plans in human and domestic animal medicine. Nevertheless, these compounds have also been misused at some point for illegal fattening in animal husbandry [11,12]. Illicit administration of thyreostats causes weight gain, which is induced by water retention in the gastro-intestinal (GI) tract and the edible tissues, through blockage of the iodium oxidation pathway, decreasing thyroid hormone production. This shrewd technique of fattening was most of all profitable to the producer, since this type of weight gain was not correlated to feed conversion. Irrefutable the consumer was the victim of such practices since meat of lower quality was produced selling water for the price of meat [13-14]. Furthermore, the residues of thyreostatic drugs may cause teratogenic and carcinogenic effects [15-18]. For example in Spain, the consumption of meat contaminated with TAP has been related to an increased incidence of aplasia cutis, a characteristic scalp defect [16]. For these reasons, the use of thyreostatic drugs for animal fatting purposes has been prohibited in Belgium since 1974 [19]. Later on the European Union (EU) followed, setting a general agreement on the ban of these substances in place, in 1981 [20]. Within the framework of residue control of xenobiotic thyreostats, 2-thiouracil, 6-methyl-2-thiouracil, 6-propyl-2-thiouracil, 1-methyl-2-mercaptoimidazole and 2-mercaptoimidazole are of most interest [11].

1.1.2. Dietary goitrogens

Besides the synthetic thyreostats, also naturally occurring compounds with goitrogenic effects have been described, which can affect organisms through ingestion of specific crops (dietary), respectively: the oxazolidine-2-thiones (OZTs) and thiocyanates [12,21]. Besides, these thiocyanates may be chemically synthesized as well. The thioamide group of these naturally occurring thyreostats is, just as for the synthetic therapeutic drugs, responsible for its thyroid inhibiting activities.

The origin of natural thyreostats lies in the interaction between secondary plant metabolites, called glucosinolates (GLs) (precursor), and the plant enzyme, myrosinase, which are both involved in the plant's defense against tissue damaging pests [22,23]. GLs are known to be most abundantly present in Brassicaceae (syn. Cruciferae) and related crop families [24-26].

Notwithstanding, GLs are by no means confined to the Brassicaceae family [26,27], their affluence in the Brassicaceae is however not comparable to other crops (Capparaceae, Caricaceae), who merely contain traces of these secondary metabolites. Chemically, GLs are characterized by a common chemical structure, constituted out of a β-thioglucoside, β-D-glucopyranoside, N-hydroxysulfate and a variable side chain derived from an amino acid [21,29-31], which makes them water-soluble phytochemicals [27]. Depending on the amino acid precursor GLs can be divided into three groups: (i) aliphatic GLs derived from leucine, isoleucine, valine, and methionine; (ii) aromatic GLs derived from phenylalanine and tyrosine; and (iii) indolic GLs derived from tryptophan. The biosynthesis of GLs proceeds through three separate phases, the chain elongation of selected precursor amino acids, the formation of the core GLs structure, and finally modifications of the side chain [32].
Nowadays about 200 different GLs have been identified and described in literature [33-35,27], but still now and then new representatives are discovered [36].

GLs can form degradation products resulting from hydrolysis through within-plant or plant-bacterial interactions. In case of within-plant formation, the physically separated GLs in the idioblast cells and the plant-derived myrosinase enzyme (ß-thioglucosidase, thioglucoside glucohydrolase, *E.C 3.2.1.147*) in the parenchymatous tissue [37] are freed upon cell disruption (e.g. chewing), causing formation of various degradation products (oxazolidine-thiones (5-vinyl-2-oxazolidinethione; 5-vinyl-1,3-oxazolodine-2-thione (5-VOT)), nitriles, epithionitriles (R=alkene), thiocyanates, isothiocyanates (goitrin), thiourea) [22,24,33] through enzymatic hydrolysis dependent of specific pH conditions [22,28] (Figure I.3). Secondly, glucosinolate enzymolysis can also be induced by microbiota of the GI tract [21,38,39]. In humans different intestinal

microbial strains were found capable of metabolising GLs, e.g. *Bacteroides thetaiotaomicron, Bacteroides vulgates, Bifidobacterium (B.) adolescentis, B. longum, B. pseudocatenulatum, Escherichia coli* and *Lactobacillus agilis* strain R16 [40-43]. Based on these findings the theory grew that natural occurrence of thiouracil could possibly be induced by the interaction between intestinal bacteria and GL-rich feeds.

Upon hydrolysis, a glucose molecule and an unstable aglucone moiety are formed, rapidly undergoing spontaneous rearrangement, eliminating sulphur, to yield a variety of biological active compounds, e.g. isothiocyanates, thiocyanates, oxazolidine-2-thiones, and nitriles (Figure I.3). The specific metabolite formation depends entirely upon the pH and the nature of the GL side chain (R) [24,29,22]. At acidic pH (2-5) epithionitrile, thiocyanates and nitriles are formed. Between pH of 3-4 sulphur is split off and a nitrile is produced, which can split further to form cyanide (CN^-). At pH 5 to 9 isothiocyanates ($i\text{-}SCN^-$) are formed, from which the naturally occurring thyreostats, thiocyanates (SCN^-) and oxazolidine-2-thiones (OZTs) (e.g. 5-VOT) can be generated. This formation depends upon the structure of the variable side chain (R). At a pH above 8 and in case the R group consists of an indolic or benzenoid structure, the instable isothiocyanates may rearrange to form thiocyanates. An appropriately located β-hydroxyl group is further needed for spontaneous cyclisation to take place and oxazolidine-thiones to form [1,6].

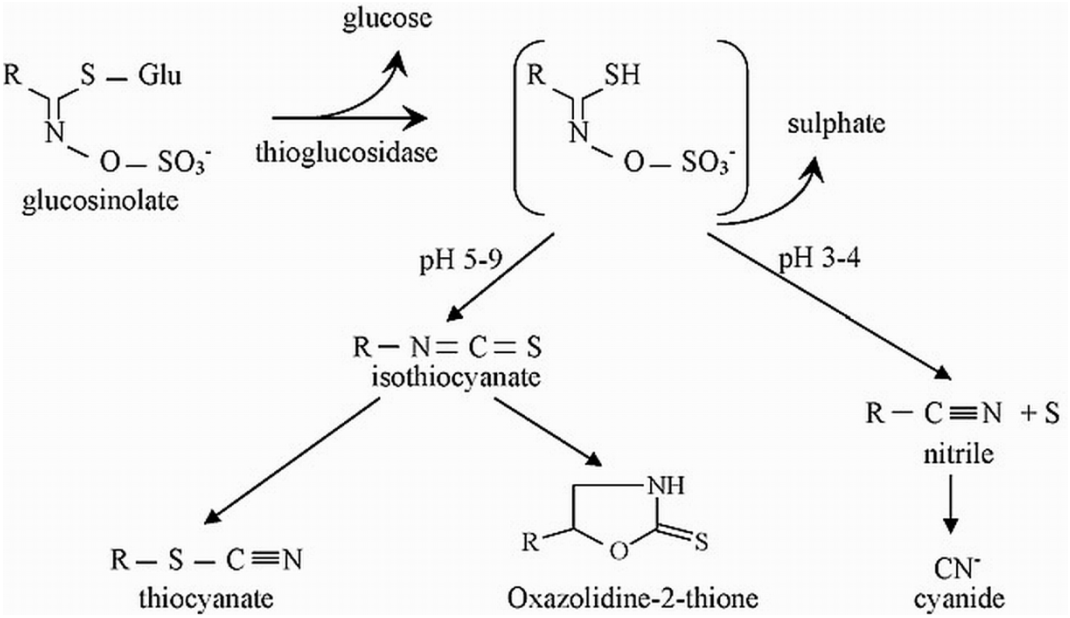


Figure I.3: Overview of myrosinase hydrolysis of glucosinolates [6].

Of the VOTs, 5-VOT or goitrin is the most commonly screened and detected analyte within the framework of residue analysis for the naturally occurring thyreostats [26,21,44-49]. Its goitrogenic activity is well know, i.e. 133 % if compared to the activity of propylthiouracil [12,46]. 5-VOT is formed from the glucosinolate progoitrin or 2-hydroxybut-3-enylglucosinolate, as a result of a myrosinase catalysed hydrolysis reaction [21,49].

1.1.3. Other goitrogenic substances

Finally, a large number of other goitrogenic molecules exist including simple monovalent anions, such as lithium (Li^+) [50-55], perchlorate (ClO_4^-) [56-59], SCN^- [56,60,61], and nitrates (NO_3^-) and a large amount of complex anions like monofluorosulfonate, difluorophosphate and fluoroborate. All these components inhibit the iodide transport into the thyroid gland, depressing iodide uptake and subsequent thyroid hormone formation. Besides, also other substances, like sulphonamides [7,62-65], ketoconazole and acetylsalicylic acid [1], phenylbutazone, interferon-γ (IFN-γ), interleukine-2 and minerals (calcium, colbalt) [1] may exert a thyreostatic action [66-67]. Some of these components inhibit thyroid hormone secretion, but others act through yet unknown mechanisms [1]. Moreover iodide, which is an essential element for the synthesis of thyroid hormones, at high concentrations can also modulate the function of the thyroid gland by producing a mild and transient inhibition of organic binding of iodide and hence of hormone synthesis. This inhibition is known as the Wolff-Chaikoff effect [68,61].

1.2. Implications of thyreostats in animal production: legislation duality

The goal of up to date legislation concerning food safety is maintaining a healthy food chain with as little harmful residues as possible. Therefore, research lies at the base of food safety legislation. For thyreostats, a risk exists that when administered to livestock, residues could remain in their edible tissues or produce (eggs, milk), which are predestined for human consumption. Based on the potential of causing deleterious effects on human health, the EU has made a strong statement by banishing them from animal production [18,20].

Subsequently, the detection of thyreostat abuse is strictly regulated, with a minimum required performance limit (MRPL) of 100 µg L^{-1} to be achieve by the applied analytical techniques (urine). Over the years, however, a great evolution in mass spectrometrical techniques took place [6], causing TU concentration at low-level

concentrations to emerge, which were far below the set MRPL. This brings about a legislative duality since at the European level a zero-tolerance is still in place for thyreostat residues in food products from animal origin, but research has shown that also natural occurrence of thyreostats is possible originating from feed (<10 ppb) [69]. This discrepancy, between the harsh legislation at hand and the actual prevalence of thyreostats in national control plans, as well as, the repercussions subsequent to non-compliancy need a more tailored approach. Therefore this paragraph presents the most relevant legislation and latest up-dates concerning thyreostatic drug residues in matrices from animal origin.

Belgium was the first European country to prohibit substances with thyreostatic action in 1974 [19]. The EU only adopted this legislation 7 years later in 1981, as Council Directive 81/602/EC [20]. Later on, Council Directive 85/358/EC [70] amended Council Directive 81/602/EC [20], to guarantee a uniform application for detection and monitoring of thyreostatic drugs in all member states. Council Directive 96/22/EC [71], the revision of Council Directive 81/602/EC [20] described the prohibition on the use of certain substances with hormonal or thyreostatic action in stock farming. Additionally, it promulgates that member states have to prohibit the import of meat from third countries hailing from animals treated with in the EU prohibited substances.

For an effective control of residues in animal matrices, adequate measures had to be taken and up-to-scale detection methods needed to be developed. Therefore, measures to monitor (e.g.: establish (self-) monitoring plans, responsibilization of operators, measures in case of infringement) the residue control of certain substances (listed in Annex I), e.g. thyreostats in live animals and animal products were described by Council Directive 96/23/EC [72]. Two groups of substances, Group A and Group B, were included here, in Directive 96/23/EC [72] listed in Annex I, based on Commission Regulation (EU) No. 37/2010 [73]. Group A substances comprise substances with hormonal or thyreostatic action, resorcyclic acid lactones, stilbenes and analogues, steroids, β-agonists (Directive 96/22/EC) [71] and banned veterinary drugs (included in Annex IV of Council Regulation (EU) No. 2377/90) [74]. Group B contains other veterinary drugs (antibacterial components (e.g. sulphonamides), anthelmintics, anticoccidials, sedatives, non-steroidal anti-inflammatory drugs, unlicensed drugs for veterinary use etc.) and contaminants (organochlorine (PCBs) and organophosphorus compounds, mycotoxins, heavy metals, dyes etc.).

To be able to implement all the rules and regulation described in Directive 96/23/EC [72], it is necessary to attain a standardised interpretation of results in official

control laboratories. And in particular for substances that are not authorized or prohibited by the EU, the progressive establishment of a MRPL for analytical methods is crucial. For thyreostats, a suggested MRPL has been fixed at 100 µg L^{-1} or µg kg^{-1}. Additionally, in December 2007 a recommended concentration (RC) for TU, MTU, PTU and TAP of 10 µg L^{-1} or µg kg^{-1} in urine or thyroid has been proposed by the EU Reference Laboratories (EURLs) in the EURL guidance paper [75]. However, this document has no legal force of action, it can serve as a technical guidance for the laboratories performing analytical thyreostatic drugs detection for the residue control plan. This RC is considered as a more up-to-date value for setting the sensitivity level of the analytical detection method for thyreostatic drugs, even though nowadays most applied methods even perform below this concentration. Nevertheless, an adapted EU supported legislation concerning the sensitivity level for thyreostat detection is required for further standardization. Therefore Council Regulation (EC) No 470/2009 introduces the concept of reference points for action (RPA) [76]. This is the lowest residue concentration of a residue for which no maximum residue limit has been set, and which can be quantified with an analytical method validated in accordance with Community requirements. So, in case the RPA is exceeded the EU country will reject the consignment, as it is not legally marketable at that point. Lately the European food safety authority published a guidance paper with a step-wise approach to establish RPAs taking into consideration factors such as analytical capability, toxic potential and pharmacological activity of the substance in question, and including the identification of the Reasonably Achievable Lowest Limit of Quantification (RALLOQ), the establishment of a Toxicological Screening Value (TSV) and the derivation of a Toxicologically Based Limit of Quantification (TBLOQ) [78]. So far, no RPA has been set for thyreostats, but if so the MRPL or RC should be declined [78].

To ensure a harmonized implementation of Directive 96/23/EC [72], Commission Decision 2002/657/EC [79] lays down the technical guidelines and performance criteria for residue control. Within this Commission Decision (2002/657/EC) [79], a system of identification points (IPs) is introduced in order to interpret the obtained data (chromatograms, spectra) when detection methods are used other than full-scan mass spectrometer (MS) techniques. This system is based on the number and the ratio of the ions in the obtained MS spectrum. For the confirmation of the banned substances, listed as group A (e.g. thyreostats), a minimum of four IPs is required. Since the implementation of the 2002/657/EC criteria [79], few studies describe the applicability of these guidelines for determination of thyreostats in urine and thyroid [80-83]. Parameters that can be evaluated during the

validation procedure are: selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, stability, repeatability, reproducibility, decision limit (CC_α) and detection capability (CC_β).

The occurrence of thiouracil in urine of livestock can be characterised as sporadic and unpredictable. In Table I.1 the overall prevalence of thiouracil in urine has been described based on collected data from European national control plan campaigns (Belgium, France, Netherlands, Poland, Norway and the United Kingdom) throughout the period 2010-2012 [84]. During that period Belgium primarily collected bovine urines of which 7.0% were above the 10 µg L^{-1} RC range. This number rose to 10.2% when zero-tolerance was applied (> CC_α).

Table I.1: Representation of the results of the national control campaigns in various EU countries from 2010-2012, showing number of samples collected [84] *(CC_α=3.9 µg TU L^{-1})*

	Porcines	**Bovines**	**Small livestock**
Member states	♀+♂	♀+♂	♀+♂
The Netherlands	139	273	0
Belgium	4	244	0
France	0	1718	0
United Kingdom	2	871	5
Norway	124	432	79
Poland	385	356	1
The Netherlands	139	273	0
Belgium	4	244	0
n (TOTAL)	654	3894	85
Mean *(µg TU L^{-1})*	1.5	1.7	1.6
Median *(µg TU L^{-1})*	0	0	0
s.d. *(µg TU L^{-1})*	2.8	4.6	3.9
Min *(µg TU L^{-1})*	CC_α	CC_α	CC_α
Max *(µg TU L^{-1})*	26.6	145.0	27.0
95% percentile *[95% C.I.] (µg TU L^{-1})*	7.4 [6.4-8.4]	8.1 [7.2-8.9]	7.3 [4.3-17.0]
99% percentile *[95% C.I.] (µg TU L^{-1})*	13.5 [10.5-17.0]	18.2 [15.5-20.0]	n.a.
% n > 10 ppb	2.3%	3.6%	2.4%
% n > CC_α	22.9%	12.4%	14.1%

n.a.= not available, only 85 animals were sampled so no 99% percentile was predicted.

2. THYROID HORMONES

The thyroid gland is one of the smaller organs of the mammalian organism, but notwithstanding, this endocrine gland has quite an important impact on the general metabolism of vertebrates [85]. Moreover, it is the target organ for the effects of therapeutic and dietary goitrogens.

2.1. Thyroid anatomy and physiology in animals

The thyroid gland is a small organ (only 0.20% of the body weight) situated in the neck region, just below the larynx, located ventrally on the trachea [1,86,87]. Depending on the species, anatomy varies but in most mammals the thyroid consists of a bilobed structure. In ruminants (D) and horses the lobes are joined by a tissue bridge, called isthmus (B) (Figure I.4). In pigs the lobes closely joined with an isthmus consisting of a small, central pyramidal lobe as part of that structure. The thyroid originates from the branchial arches of the foetus, which starts migrating to the neck region during early foetal development [86]. Occasionally, these cells do not migrate properly and ectopic thyroid tissue remains e.g. at the tongue base.

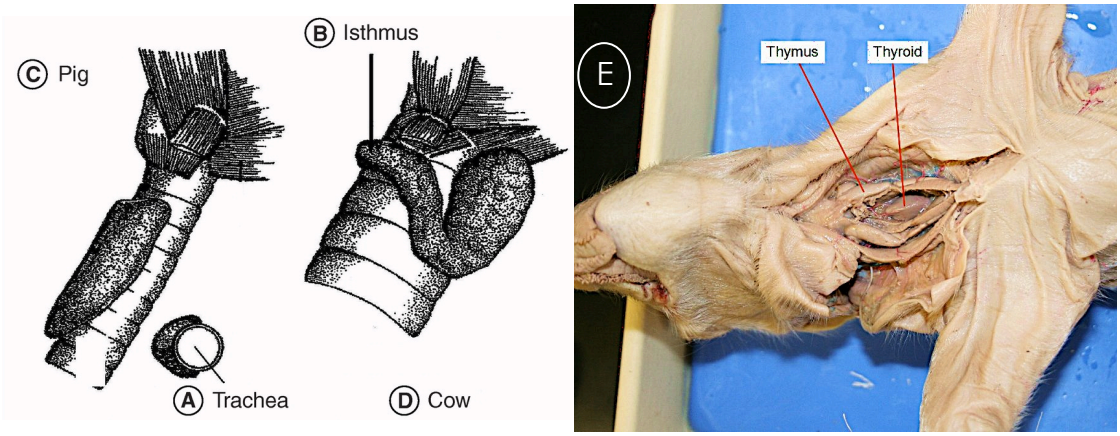


Figure I.4: Representation of the topographical anatomy of the thyroid gland in pig (C) and cow (D) with representation of the subtracheal connection in pigs (A) [1]. Dissection of a fetal pig showing the thyroid *in situ* (ventral aspect) (E) (©faculty.clintoncc.suny.edu).

The thyroid tissue consists of interconnected globular sacs, called follicles, which are separately enfolded by a single layer of epithelial cells. In response to the down- or up-regulation of the thyroid gland, these cells can respectively become cubical to cylindrical or even flattened. This is due to the viscous protein-rich fluid in the lumen of

the follicles also called colloid, which decreases with up-regulation of the gland. The follicles themselves are surrounded by a basal membrane beneath which an elaborate capillary network is situated to transport nutrients and the produced thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) (Figure I.5). In the surrounding connective tissue, the thyroid C-cells (parafollicular cells) are located, which produce the calcium-regulating hormone, calcitonin. In most animals spherical clusters of parathyroidal cells are also embedded in the thyroid tissue, which in combination with the C-cells regulate Ca^{2+} concentrations in the extracellular tissues [1,86,87].

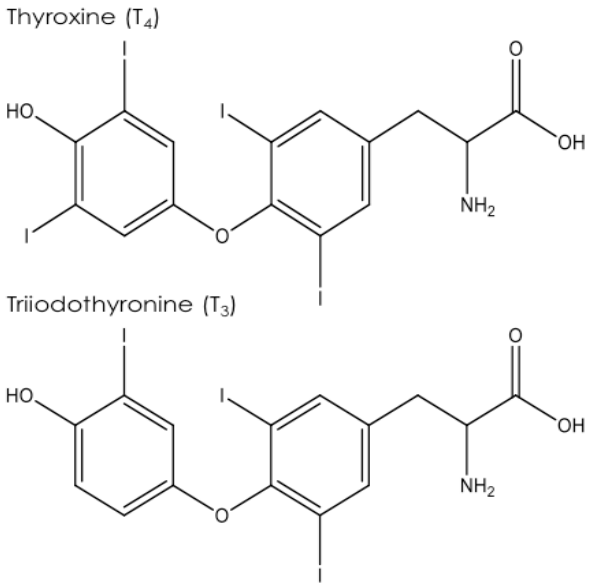


Figure I.5: Chemical structures of the thyroid hormones, T_4 (3,5,3',5'-tetraiodothyronine) and T_3 (3,5,3'-triiodothyronine).

The thyroid hormones, T_3 and T_4 (Figure I.5) are the only iodine containing water insoluble hormonal compounds in vertebrates [1]. Therefore, ingested iodine (I^-) is absorbed in the small intestine and transported directly to the thyroid gland through the bloodstream, entering the organ passively through a Na^+/I^- symporter activated by the gradient of a Na^+/K^+ adenosine triphosphatase (Figure I.6). In the follicle cells, iodine will be oxidized ($I^- \rightarrow I_2$) by thyroid peroxidase (TPO) in the presence of hydrogen peroxide (H_2O_2). TPO is either located in the apical membrane of the cell or attached to it, thus providing the oxidized iodine at exactly the point in the cell where the thyroglobulin (Tg) molecule issues forth from the Golgi apparatus and through the cell membrane into the stored thyroid gland colloid. The more reactive iodide (I_2) binds to the double-bounds of the tyrosine residues on Tg, a colloid protein, forming monoiodotyrosine (MIT) and diiodotyrosine (DIT). These building blocks either dimerize

(T_4) or couple (DIT+MIT= T_3 (or less common rT_3)) to form both thyroid hormones catalysed by TPO and H_2O_2 while remaining bound to Tg and stored in the lumen of the follicle (Figure I.6). Overall only 10% of the tyrosin residues on Tg molecules are iodinated; nevertheless the colloid contains enough thyroid hormone to provide the normal requirements of the body for 2 to 3 months [1,86,87].

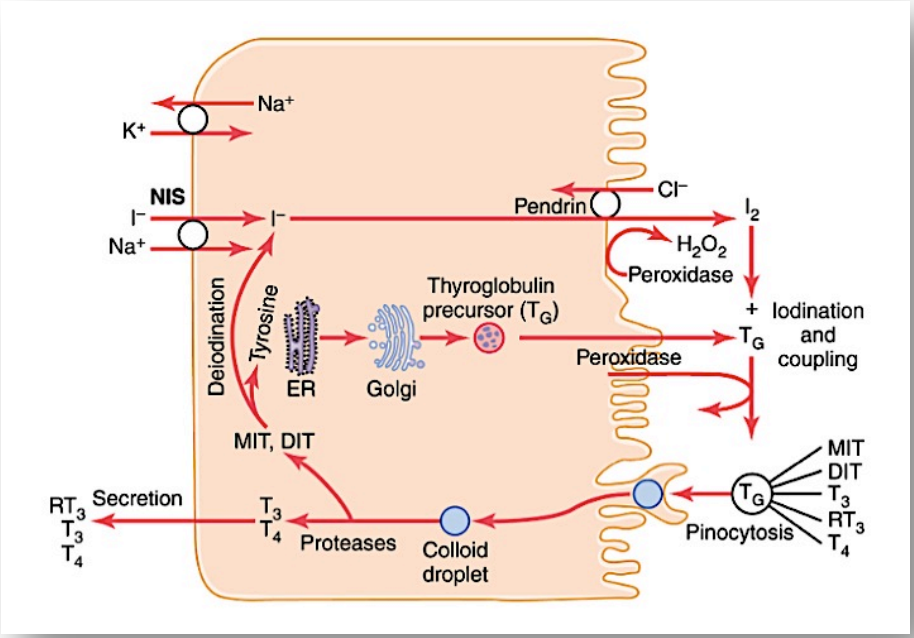


Figure I.6: Schematic representation of thyroid hormone synthesis and secretion (NIS: sodium iodied symporter; TPO: thyroid peroxidase; MIT: monoiodotyrosine; DIT: diiodotyrosine) (©2014 Quizlet.com).

Secretion of the formed hormones occurs through endocytosis of small colloid vesicles of iodinated Tg that fuse with lysosomes causing proteolysis of Tg, thereby recapturing MIT and DIT (deiodination) and releasing T_3 and T_4, which diffuse across the basal membrane into the interstitial fluid and into the capillaries (Figure I.6) [86,88]. T_4 is more abundantly produced than T_3, since the latters' concentration is 50- to 60-times lower in total serum [86]. Moreover, in the bloodstream the majority of T_4 molecules are bound to carrier proteins (thyroxine binding globulin (TBG), albumin, thyroid binding prealbumin) with a remaining 0.03 % of T_4, which occurs unbound. For T_3 approximately 0.3% is free, with the remainder bound. Between both, free and protein-bound fractions, an equilibrium is installed. The small circulating free thyroid hormone fraction is solely responsible for interactions with target cells and the

subsequent generation biological response [1,86,88]. Furthermore, only T_3 is directly biologically active whereas T_4 first needs deiodination. Deiodinase (Type I and II) causes 5'-deiodination of the outer ring of T_4, which accounts for the majority of circulating T_3 [88]. Type I deiodinase is responsible for the majority of T_4 to T_3 transformation and is found in peripheral tissues (liver and kidney). Type II deiodinase is found in the brain, pituitary gland, and brown adipose tissue and primarily converts T_4 to T_3 for intracellular use. Type III deiodinase, which is mostly found in placenta, brain, and skin, can along with Type I also lead to the inactive form of T_3, i.e. reverse T_3, which has a key role in the inactivation of thyroid hormones. Both, rT_3 and T_3, can be further deiodinated in the liver and are sulfo- and glucuronide-conjugated before excretion into the bile. Besides, there is also an enterohepatic circulation of thyroid hormones as intestinal microbiota deconjugate some of these compounds and promote their reuptake [88].

The regulation of thyroid hormone secretion is an example of a negative feedback mechanism between the hypothalamus, anterior pituitary and thyroid gland (Figure I.7). The hypothalamus produces thyroid-releasing hormone (TRH) under the influence of environmental (temperature) and internal stimuli (thyroid hormone imbalance) [86].

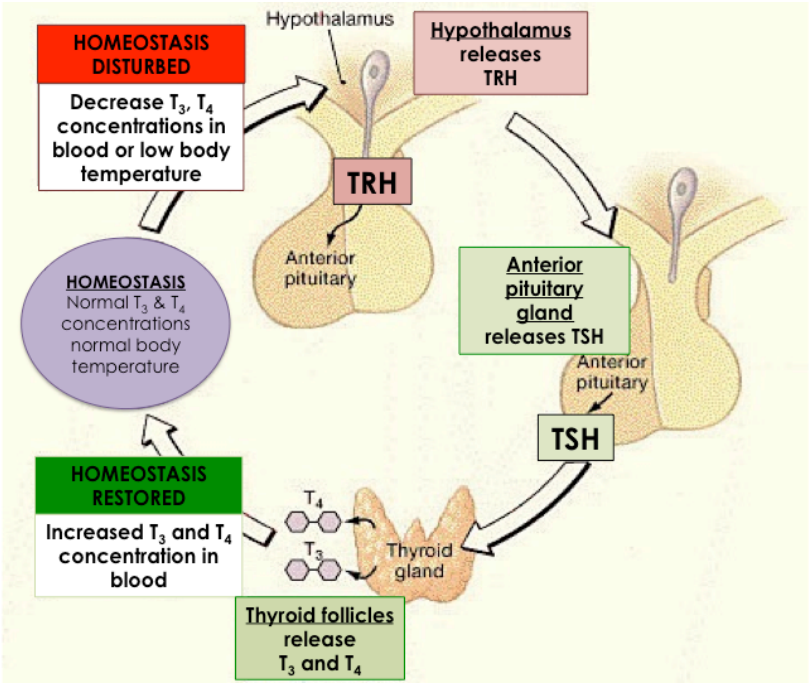


Figure I.7: Overview of the negative feedback mechanism for thyroid hormone regulation (TRH: thyroid-releasing hormone; TSH: thyroid stimulating hormone) (©2014 Cram.com).

TRH in turn influences the anterior pituitary gland, which then produces thyroid stimulating hormone (TSH), activating the thyroid gland and subsequent thyroid hormone production. When homeostasis is reached, the thyroid hormones exert a negative influence on all the above contributors in the chain in order to cease stimulation. If TSH production is increased over a long period of time the number (hyperplasia) and volume (hypertrophy) of the follicle cells will increase, forming a goiter or non-neoplastic and non-inflammatory enlargement of the thyroid gland [89].

2.2. Function of the thyroid hormones

Within the bloodstream only the free fraction of thyroid hormones, T_3 and T_4, can be actively transported into the cell. First, T_4 will be deiodinated to T_3 in the cytosol, whereas T_3 can directly proceed with binding to a nuclear thyroid hormone receptor on DNA. Second, because T_3 has a higher affinity for the receptor and induces a 5-times greater effect than T_4, it is primarily T_3 that mediates the biological effect. Subsequent to binding a cascade of changes occurs resulting in alteration of the synthesis of certain cellular proteins. Since these play a major role in cell metabolism, thyroid hormones can induce an effect on all types of cells, with a variety of mechanisms [86]:

Thyroid hormones will increase metabolic rates in all tissues (except in the brain, gonads, spleen), resulting in increased heat production and oxygen consumption.

Normal growth depends on adequate production of thyroid hormones, since a lack during growth causes depletion in growth hormones and subsequent impaired skeletal development.

Normal development of the central nervous system depends on normal thyroid concentrations with the most critical moments 6 months before and 6 months after birth. When shortage is sustained at those points in time, irreversible reduction of the development and maturation of the brain occurs.

The sympathic nervous system is stimulated by thyroid hormones (e.g.: heart rate increases), which results from the numeral augmentation of available epinephrine and norepinephrine receptors.

Conduction velocity of the nervous system is also increased by thyroid hormones. Therefore, a shortfall will induce slower reflexes, impaired physical and cognitive performances.

Normal gonadal function requires thyroid hormones, thereby inducing sperm count depletion (male) and irregular reproductive cycles (female) when deficiency is encountered.

2.3. Thyroid disruption and mode of action of thyreostatic agents

Various compounds may induce either an up-regulation or a down-regulation of thyroid hormone secretion. This may result in two diametrically opposite clinical pictures, called hypothyroidism (down-regulation) or hyperthyroidism (up-regulation) [90,91]. In both clinical pathologies a distinction based on the cause of the illness can be made; either primary, which is related to the thyroid gland (inflammation, tumor) itself or secondary, when the cause is due to external factors (pituitary gland, hypothalamus, dietary iodine, iatrogenic substances).

Hypothyroidism is induced by a diminished production of thyroid hormones. Symptoms include a.o. cold intolerance, animals become slow and sluggish, reluctant to move, reduced appetite, weight gain, alopecia, skin infections, impaired reproduction, reduced heart rate, slow bowl movements and constipation. Goiter formation may also be associated with hypothyroidism, e.g. due to lack or excess of iodine in feed, plant-derived goitrogenic substances, tumor or inflammation of the thyroid, inherited enzyme defects in the biosynthesis of thyroid hormones [86,89].

Therapeutically, most cases of hypothyroidism can be controlled through administration of T_4, compensating the hormone paucity. This disorder is most common in dogs, and only rarely develops in other species, including cats, horses, and other large domestic animals [89].

Non-neoplastic and non-inflammatory goiter can develop in all domesticated animals [89]. Goiter caused by iodine deficiency is most common in pigs, lambs, calves and foals. In foals, a congenital hypothyroidism and dysmaturation syndrome has been described when pregnant mares graze plants that contain goitrogens, nitrate or are fed diets either deficient in or containing excessive amounts of iodine [89]. Young animals are born with thyroid lobes at least twice the normal size, soft and dark red, grossly enlarging the neck area. In severe cases total or partial alopecia (lack of hair especially pigs and wool in lambs), myxedema (thickened, flabby,

edematous skin) can develop. The mortality rate is high in these species, with majority of offspring born dead or dying within a few hours of birth [87]. Only in mildly affected cases of iodine deficiency administration of iodized salt might resolve the goiter and clinical symptoms [89]. Undoubtedly, this is an issue that if not controlled might lead to economic losses, from the loss of offspring and breeding animals due to difficulties at birth.

In sheep, cattle, goats and pigs a familial dyshormonogenetic goiter has been described, an inherited autosomal recessive trait, causing an enzyme defect in the thyroid hormone biosynthesis [89]. Goiter in adult livestock, however, is usually of little significance in comparison and the general health of the animal is not impaired [87].

In contrast, increased thyroid hormone secretion leads to symptoms of hyperthyroidism, involving amongst others: increased appetite, weight loss, polydipsia, increased excitability, nervousness, reduced physical performance, tiring easily, increased heart and breathing rate, increased bowl movements and diarrhea [86]. This is quite a prevalent illness in middle-aged to older cats mostly due to (>70%) functional thyroid adenoma and only rarely occurs in dogs where it is mostly due to thyroid carcinoma. Disorders of the thyroid gland are clearly well known in companion animals but less so in livestock. In livestock, nutritional iodine deficiencies (due to iodine-deficient diet or geographical location) have been of greater importance than thyroid-gland diseases, particularly in the iodine-deficient areas [86].

When treating hyperthyroidism, several strategies can be followed, including feeding an iodine-deficient diet, chronically administering anti-thyroid drugs (e.g. methimazole in cats), destroying dysfunctional thyroid cells with radioiodine therapy or finally surgically removing the gland (thyroidectomy) [89].

The primary effect induced by thyreostatic drugs is the inhibition of thyroid hormone synthesis by interfering with the TPO iodination of tyrosine residues in Tg. PTU (6-propyl-2-thiouracil) and methimazole (1-methyl-2-mercaptoimidazole, TAP) are the antithyroid drugs mainly used in the United States. In Europe and Asia mostly methimazole dominates whereas carbimazole (methimazole analogue) is mostly administered in the United Kingdom [92].

Table I.2: Overview of the mode of action of various dietary and therapeutic goitrogens [89,92,93].

Compounds with thyreostatic effects	Mechanism(s) of action
Thiocyanate, Perchlorate	Inhibition of the iodine-trapping mechanism
Sulphonamides, Thiourea, Methimazole, Aminotriazole	Blockage of organic binding of iodine and coupling of iodothyronines to form T_4 and T_3
Lithium, Excess of iodide	Inhibition of thyroid hormone secretion by an effect on the proteolysis of active hormone from the colloid
Drugs of the central nervous system (e.g., phenobarbital, benzodiazepines), **Calcium channel blockers** (e.g., nicardipine, nifedipine), **Steroids** (e.g., spironolactone), **Retinoids, Chlorinated hydrocarbons** (e.g., chlordane, DDT, TCDD), **Polyhalogenated biphenyls** (e.g., PCB, PBB), **Enzyme inducers**	Increase the peripheral metabolism of thyroid hormones through induction of hepatic microsomal enzymes
Amiodarone (Anti-arrithmicum), **Iopanoic acid** (Hyperthyroidism treatment in cats)	Inhibit the 5'monodeiodinase, which converts T_4 in peripheral sites (e.g., liver and kidney) to biologically active T_3
Natural goitrogens (from e.g., rapeseed, kale, turnip, cabbage)	Inhibition of the iodine-trapping mechanism and disrupt signalling across the thyroid's cell membranes
Iodine-deficient diet	Inhibition of iodination of Tg

For propylthiouracil, blockage of the conversion of thyroxine to triiodothyronine within the thyroid gland and in peripheral tissues has been reported, but this effect seems to be clinically insignificant. Some antithyroid drugs may also induce immunosuppressive effects [92], which can be beneficial when treating autoimmune thyroid disorders. Besides specific thyroid inhibiting drugs, certain chemicals also induce a thyreostatic effect of which some examples are shown in Table I.2.

A consistent finding with all of these goitrogens is the chronic hypersecretion of TSH due to the lack of thyroid hormone production, which by receptor-mediated events places the thyroid gland at greater risk of developing tumors because of constant stimulation [8].

3. BRASSICACEAE

The genus *Brassica* of the Brassicaceae or Cruciferae (cross-like shape) plant family represents important revenues for the agricultural and horticultural industry. This genus comprises all sorts of cabbages and rapeseeds.

For human consumption a large variety, e.g. cauliflower, broccoli, white cabbage, green cabbage, savoy cabbage, Chinese cabbage, watercress, mustard, and rapeseed oil, is available and included in the normal diet. Particular for this genus is the fact that all parts of the plant may be edible: root (rutabaga, turnips), stems (kohlrabi), leaves (cabbage, collard greens), flowers (cauliflower, broccoli), buds (Brussels sprouts, cabbage), and seeds (many, including mustard seed, and oil-producing rapeseed). Moreover, their high glucosinolate content has caught the attention, as well as, the glucosinolate degradation products, which for some might contribute to reduction of carcinogenesis in humans (e.g.: the isothiocyanate sulforaphane) and have health-promoting effects [94].

In recent years, the oil extracted from rapeseed (rapeseed oil) gained new interest when '00' rapeseed varieties with improved fatty acid composition became available. These '00' varieties are low not only in bitter tasting glucosinolates (GLs) but also in erucic acid, which is a C_{22} monounsaturated fat that has been shown to cause a wide variety of pathological changes in laboratory animals. Thereby, the utilization of this now healthier rapeseed oil became more relevant in the kitchen, either for cooking or as a dressing, alongside the traditional olive oil. In addition, rapeseed '00' oil displays a low content in saturated fats (7%) and a high amount of monounsaturated fats (61%) with also a good ratio (60% oleic, 20% linoleic, 10% alphalinoleic acid) between the polyunsaturated fatty acids omega-6 and omega-3 [95]. These are essential cholesterol-lowering fatty acids, which also decrease cardiovascular risks. Currently, rapeseed production in the EU member communities occupies 6.766 million hectares, up nearly 83,000 hectares from 2013, making it the predominant oil crop in Europe (Figure I.8) [96].

The high (20-50%) erucic varieties of rapeseed are not used for consumption, but find application in technical domains, for example as bio-degradable lubricating oil as an alternative to mineral oil based lubricants.

For animal feed, rapeseed and its extraction products (rapeseed flakes, cake, and coarse meal) are generated as by-products from the oil industry and the biodiesel fuel production, which is a booming business nowadays. In order for the by-product to be useful to the animal feed industry, only '00' varieties can be used.

Evidently, the manipulation of rapeseed ('0' and '00'-varieties) contributed to its widespread application in animal and human consumption nowadays, despite its anti-nutritional factors (ANFs).

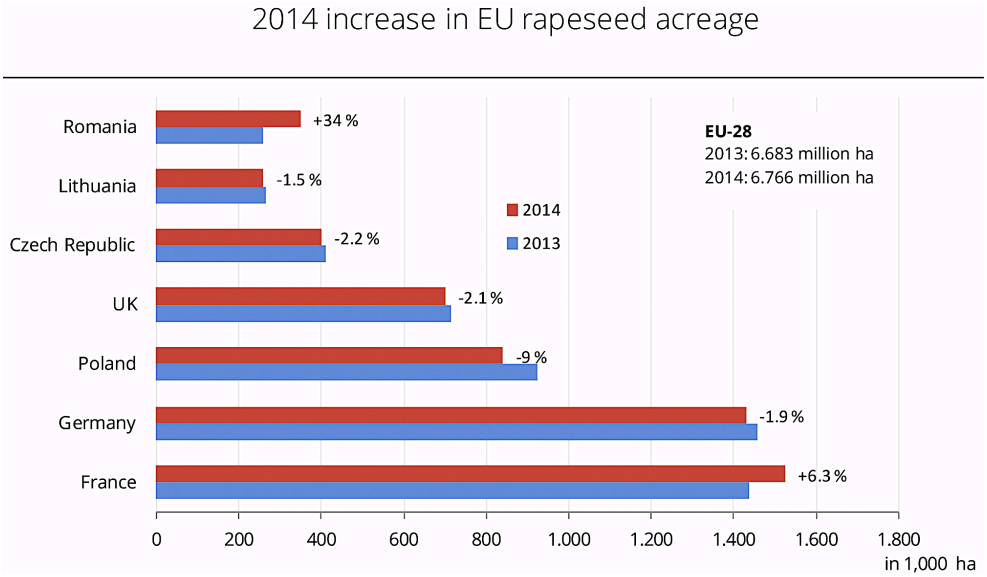


Figure I.8: Graphical representation of the amount of rapeseed acres over the predominant countries active in this business in Europe (©Coceral, the European grain and oilseed traders' association).

3.1. Anti-nutritional factors of rapeseed consumption

Rapeseed can be classified according to the fatty acid content of the extracted oil or according to the glucosinolate content of the rapeseed extraction meal (REM). From rapeseed about 42-44% of oil can be extracted, consisting of a mixture of glycerol and fatty acids (5% saturated - 95% unsaturated), with 35% of the fatty acids consisting of erucic acid [27,96-98]. After oil extraction, a rapeseed product is delivered that contains 35% proteins, a possible valuable and cheap nitrogen source for animal feed [96-98].

However, the presence of erucic acid and GLs are unwanted, as they are considered as anti-nutritional factors [21,27]. Human consumption of rapeseed oil with a normal erucic acid level (35%) is linked to possible cardiovascular and digestive problems [96]. As for the GLs a substantial intake has proven to be deleterious to animal health [21,26,99-101] not because of the GLs themselves but due to the formation of the toxic degradation products, e.g. VOTs, nitriles and (iso)thiocyanates [67]. In light of nutritional concerns, the industry turned their

attention in 1970 towards genetically engineered crops with little erucic acid present (< 2%), the 'single low' or '0' varieties of rapeseed [102-104]. A major market constraint remained however, namely the utilisation of the high-protein meal generated from the oil extraction process, still contained high glucosinolate concentrations, limiting its utilisation. Hereupon again further genetic manipulation of these crops brought relief lowering the average glucosinolate content of 166 µmol g^{-1} to 38 µmol g^{-1}. These are referred to as the 'double-low' or '00' or 'CANOLA' (CAnadian Oil, Low Acid) varieties of rapeseed [21,105], which contain little GLs and erucic acids. Modern '00'-rapeseed varieties show an even more remarkable low glucosinolate content of 8-15 µmol g^{-1} seed, thereby meeting the criteria of 25 µmol g^{-1} of glucosinolate content and the erucic acid level below 2% as set by Commission Regulation No 1035/2003 [106]. Nowadays, for all applications the 'double-low' or '00' varieties are considered as standard, for biofuel however 'traditional' rapeseed may be used with unaltered levels of erucic acid and GLs [96,97].

The set levels for undesirable substances in animal feeds have been described in the European directive 2002/32/EC [107]. Amongst these some are related to the use of Brassicaceae in animal feeds (Table I.3)

Table I.3: Selection of Brassicaceae derived undesirable substances described in directive 2002/32/EC with maximum allowed levels.

Undesirable substance in animal feed	Maximum content mg kg^{-1} relative to a feedstuff with a moisture content of 12%	Products intended for animal consumption
Volatile mustard oil	100	Feed materials (except rapeseed cakes)
as *Allyl isothiocyanate*	*4000*	Rapeseed cakes
	1000	Complete feeding stuffs for cattle, sheep, goats (except young animals)
	500	Complete feeding stuffs for pigs (except piglets) and poultry
	150	Complete feeding stuffs
Vinal thiooxazolidone (Vinyloxazolidinethione)	1000	Complete feeding stuffs for poultry
	500	Complete feeding stuffs for young laying hens
Indian mustard — *Brassica juncea* (L.) Czern. And Coss. ssp. *intergrifolia* (West.) Thell.	Trace, not quantitatively determinable	All feeding stuffs
Sareptian mustard — *Brassica juncea* (L.) Czern. And Coss. ssp. *juncea*	Trace, not quantitatively determinable	All feeding stuffs
Chinese mustard — *Brassica juncea* (L.) Czern. And Coss. ssp. *juncea var. lutea* Batalin	Trace, not quantitatively determinable	All feeding stuffs
Black mustard — *Brassica nigra* (L.) Koch 39. Ethiopian mustard — *Brassica carinata* A. Braun	Trace, not quantitatively determinable	All feeding stuffs

3.2. Animal feed

Feeds based on rapeseed consist of rapeseed derivatives from oil extraction, which produces a valuable protein-rich material, e.g. REM (rapeseed extracted meal) used as an animal diet enhancer. For consumption (animal-human), only rapeseed of the '00' variety may be used, characterized by a low erucic acid level (< 2%) and a maximum level of 25 µmol GLs per g^{-1} of seeds [106]. The REM can be produced in three commodities, rapeseed flakes, cake, or coarse meal. The type of feed formed depends on the oil extraction process. If cold compression (50-60 °C) occurs, rapeseed cake with 11-20% of fat is formed. Hot compression (80-140 °C) results in rapeseed flakes with a fat percentage of 6-10%. For the coarse colza meal, hot compression is followed by etherification and toasting with a fat percentage of 1-4% [97]. Moreover the concentration of GLs in rapeseed can be altered based on e.g. season (autumn>winter foliage) and climate (drought, temperature) [108]. This might explain the variation of glucosinolate content and composition of the meal between different producing countries.

However, with the lowered glucosinolate content, the total dietary intake of rapeseed extraction products remains restricted, due to toxic degradation products such as OZTs, nitriles, and thiocyanates, which can still form. The level of tolerance for this appears to be species and age dependent [21]. Ruminants exhibit a tolerance level of 1.5 to 4.22 µmol of total glucosinolate content g^{-1} diet, whereas pigs are severely more sensitive with 0.78 µmol g^{-1} diet. Ruminants are less affected than monogastric animals because of the microbial degradation, which already occurs in the forestomach causing transformation of GLs and of their metabolites early in the digestion process [109]. Young animals (piglets) are also more sensitive than older. The ANFs (bitterness, iodine inavailability, mutagenicity, teratogenicity, goitrogenicity, hepatotoxicity, nephrotoxicity) induced by glucosinolate intake have an overall negative effect on the animals' growth and production (milk, meat weight gain). In order to counter act the ANFs from rapeseed meals some treatments have been described to improve REM quality, like: water extraction, heat (certain time, temperature) and $CuSO_4$ treatment [21]. Even though treating rapeseed meal is an option, it is not always economical, which on the other hand iodine supplementation remarkable is, with promising results in pigs and ruminants [21]. In conclusion administration of rapeseed meal seems no longer a problem as long as a suitable technology is adopted to minimize deleterious effects [21].

4. ANALYTICAL METHODOLOGIES

4.1. Principles of LC-MS based analysis

Within this thesis the main analytical methodologies are based on LC-MS techniques, therefore only these will be expounded here.

An important part of the analysis of biological samples (e.g.: urine, serum, faeces and muscle) lies in the pre-treatment, which is an absolute requirement for eliminating/reducing interfering substances in any matrix. Implementation of certain statistical tools, like fractional factorial designs [110-112], makes optimization of extraction procedures much easier nowadays. Based on a select number of tests, information is gathered and statistically processed making the influential factors apparent in order to subsequently optimize them. These methods are much more efficient then the on-at-the-time approach optimisation procedures.

Mass spectrometrical analysis, requires hyphenation with separation techniques, either in the gas phase (gas chromatography (GC)) or liquid phase (liquid chromatography (LC)). Over the years the latter became the predominant technology in residue analysis since around 80% of analysed components are not sufficiently volatile or stable in the gas phase to be separated by GC [113]. Furthermore, the development of more performant LC separation columns, decreasing the particle size of the columns' packing material, introduced high performance liquid chromatography (HPLC). HPLC was always a compromise between particle size causing inherently higher backpressures and the resilience of the columns packing material. Therefore, Ultra-high pressure liquid chromatography (UHPLC) columns arose on the market increasing speed, sensitivity, and resolution by working with sub-2 micron particles [114-117]. These columns can sustain 15,000-19,000 psi [113]. Analytical pumps capable of operating at these ultrahigh pressures while delivering solvents smoothly and reproducibly in both gradient and isocratic separation modes were therefore made available [114,117]. With all these advantages UHPLC technology seems to have a bright future [118], moreover the market share of HPLC applications is also rising quickly with estimations of a 4 billion USD turnover by 2017 [113].

In LC, analytes are separated based on their differential affinity between a solid stationary phase (the column packed with particles) and a liquid mobile phase. To ensure optimal chromatographic separation, a proper static phase has to be selected based on the physico-chemical properties of the target analytes. The current stationary phases available on the market are characterised by a variable set

of particle active groups (C_{18}, resin ($SO_3^-H^+$)) available in different particle sizes (e.g.: 15 to below 2 µm) to separate compounds e.g.: reversed phase chromatography, ion exchange chromatography (e.g. amines or metal ions), hydrophilic interaction chromatography (highly polar water soluble analytes) [113].
Once the analytes have been separated in time, the liquid phase will need to be evaporated and ionised to allow mass-spectrometrical detection of the molecule or fragments based on mass-to-charge (*m*/z) ratio, measuring the abundance of the ions under vacuum conditions. In residue analysis, mass spectrometry has become the detector of choice for chromatographic separation [118,119]. A mass spectrometer can be divided into three components, the ion source, the mass analyser and the detector. The power of this technique lies in the fact that the mass spectral data generated provides valuable information about the molecular weight, structure, identity, and quantity with a high degree of confidence and selectivity.

Earlier LC-MS systems were only successful in the detection of a limited number of compounds due to the limitations of the ionisation interface (a.o. direct liquid injection (DL)) [120]. Target analytes in biological samples are mostly non-volatile and potentially thermally labile, but still needed to be presented in the gaseous phase for analysis, which was the challenge of hyphenating of LC with MS [113,121]. In the case of DL the mobile phase molecules are not separated from the targeted compounds, causing a large low-molecular-weight solvent background signal, and subsequent ionisation is performed under vacuum conditions [120]. Over the years evolution towards other interface techniques like, thermospray (TS), which added heat and particle beam (PB), which desolvated at near-atmospheric pressure [120], broadened the application potential, but still these methods lacked one or more critical aspects that LC demanded (e.g.: sensitivity, applicability and stability) [120]. Therefore, with the introduction of atmospheric pressure ionisation (API) techniques, the number of compounds successfully analysed by LC-MS greatly expanded (large biomolecules, pharmaceutical drugs, metabolites) [121]. In API, the analyte molecules are ionised first at atmospheric pressure, next the ions are mechanically and electrostatically separated from neutral molecules. Nowadays, common API techniques are: electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), and atmospheric pressure photoionisation (APPI).

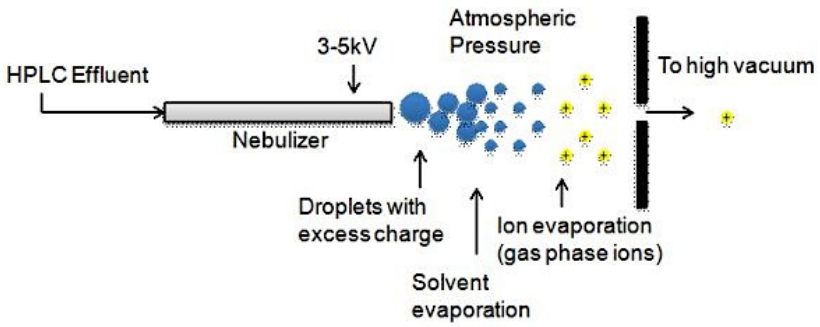


Figure I.9: Electrospray ionisation (ESI) technology [122].

During this study, all performed analyses relied on ESI (Figure I.9), which is referred to as a soft ionisation technique in the sense that a very little residual energy is retained by the analyte, and generally no fragmentation occurs upon ionization [121,123]. To generate gas phase ions from a liquid-phase sample the LC eluent is nebulized at atmospheric pressure and sprayed across a high potential difference of a few thousand volts from the capillary needle to the orifice of the interface [113]. Heat and gas flow desolvates the charged droplets, causing the droplets to shrink, subdivide, and eventually the repulsive force between ions with like charges exceeds the cohesive forces and non-fragmented ions are ejected into the gas phase. These charged ions are attracted to and thus proceed to the mass analyser. The heated ESI (HESI) interface is even better capable of mobile phase dissolvation, due to thermal desolvation assistance in the capillary needle [113,124].
During APCI, the LC solution is sprayed into a quartz heater jacket (typically 250 °C-400 °C) creating a vapour, which is then passed over the corona discharge needle creating ions through gas phase ion-molecule chemical reactions [113]. Positive ions are formed through proton transfer, while negative ions are formed through electron transfer or proton loss [113,124].

During this study, two types of mass analysers hyphenated with LC and UHPLC were utilised, respectively, an ion trap (LTQ, Thermo Fisher Scientific) and a triple quadrupole (QqQ, TSQ Vantage, Thermo Fisher Scientific) mass spectrometer.

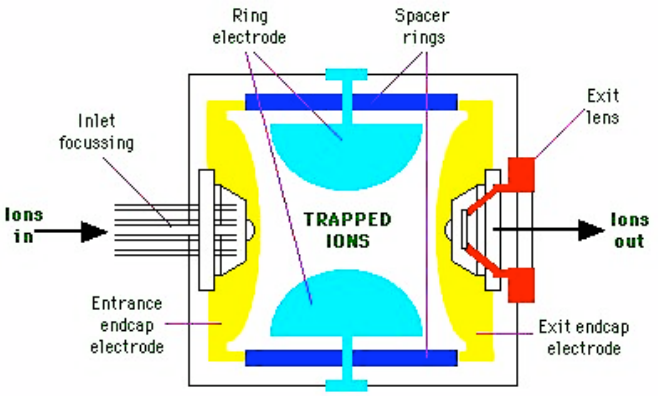


Figure I.10: Schematic overview of an ion trap mass analyser (LTQ) (©Vinaquips.com).

The ion trap mass analyser (LTQ) also referred to as the quadrupole linear ion trap relies on the effect of oscillating electric fields contained by three hyperbolic electrode (entrance, ring and endcap) arrangements (Figure I.10). The applied fields allow the ions to be trapped for extended periods of time and manipulated (consecutive tandem mass spectrometry (excitation, fragmentation and detection of ions)) in the process of an extended time domain [113]. The exact path of the ions is dependent on the voltages applied and their individual mass-to-charge (*m*/z) ratios. After trapping the precursor ions, collision activated dissociation (CAD) is applied to generate structural information [113]. For detecting the ions, the magnitude of the direct current (DC) and radiofrequency (RF) potentials are increased as well as the RF, forcing the ions to become unstable and to leave the trap through the exit end-cap towards the detector [124,125,113].

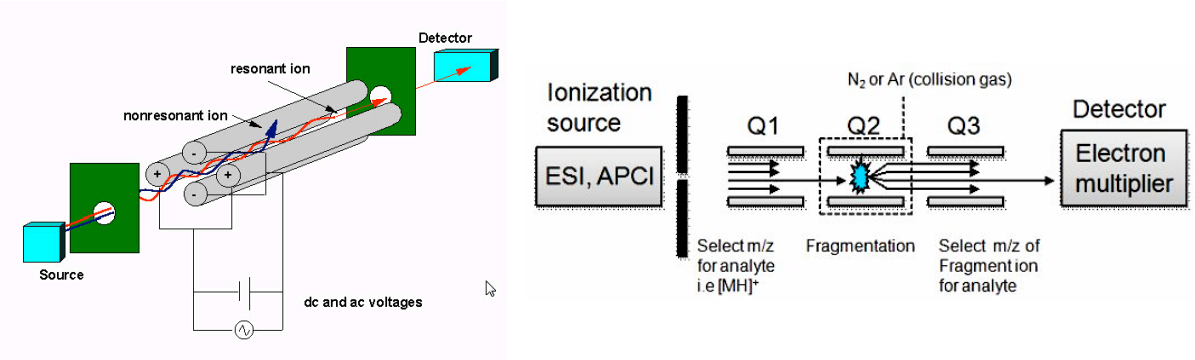


Figure I.11: Scheme of a quadrupole mass analyser (TSQ, QqQ) [122].

A quadrupole mass analyser (TSQ, QqQ) consists of three subsequent quadrupoles each composed of four parallel hyperbolic metal rods to which both a DC voltage

and an oscillating RF voltage is applied (Figure I.11). Two opposite poles are positively charged and the other two negatively, and their polarity switches throughout the analysis. The precisely applied voltages determine the trajectory of the ions down the flight path between the four rods. Only those ions with a specific *m*/z value will resonate along the field and complete the trajectory across the quadrupole. Non-resonant ions will deflect form the central path, subsequently colliding with the rods and be lost. By quickly switching the voltages, ions of one mass after another will take a steady path along the rods and be detected. This creates a 'mass filter' [113]. The second quadrupole, the collision induced dissociation (CID) cell, allows fragmentation of ions. For quadrupole mass analysers different types of MS/MS experiments are possible. The four most common types used are namely product ion scans, precursor ions scans, neutral loss scans, but also selected reaction monitoring (SRM), which is considered as the most sensitive and quantitative operating mode for MS/MS experiments [124]. And therefore the latter has been applied in our study concerning the quantitative analysis of thyroid hormones in bovine serum (cfr. *CHAPTER V*).

The detection of the ion trap and QqQ mass spectrometer is achieved by an electron multiplier, used to detect the presence of ion signals emerging from the mass analyser. The basic physical process that allows an electron multiplier to operate is called secondary electron emission. When ions hit a surface, it causes the electrons in the outermost area of the atom to be released, which are known as secondary electrons. These electrons are emitted and then accelerated through an electric field, which is generated by applying the proper voltage to the surface of the tube. The electric field forces the emitted electrons to hit the following wall, and these electrons, like the ion, also cause electrons to be emitted. This cascade continues until enough electrons are emitted to create a measurable current [113].

4.2. Analysis of thyreostats in animal matrices

In the early days, suspicion of thyreostatic abuse was based on the symptomatology of thyroid gland activity decrease coherent with the illicit administration of thyreostats in livestock. Animals administered with thyreostats would look sedated (muscle weakness and retarded tendon reflexes) and display mucous inflated skin (myxedema) caused by the deposition of mucopolysaccharides. Moreover their body weight would be increased due to water retention in the GI tract and edible tissues. Obviously, the decrease of thyroid hormones (T_3, T_4), which could be easily detected by immunological techniques, like radioimmunoassay (RIA)

or enzyme-linked immunosorbent assay (ELISA), could form an indication of thyreostat abuse [11,122]. All these parameters were rather subjective; therefore weighing the thyroid gland enlargement (goiter) was a less subjective non-analytical quantitative evaluation, which was also applicable at slaughterhouse level. Even though, false positive (above 60 g = suspected thyroid) results were reported for this technique, false negatives were almost nihil [12,14]. Besides, the histological pattern could be microscopically investigated, which in case of abuse would be altered [14,127,128].

Over the years a huge analytical evolution has taken place in the analysis of thyreostats in animal matrices, which has been reviewed by Vanden Bussche *et al.* (2009) [6]. Briefly, in the early days the chemical analysis of thyreostats from animal matrices consisted of a colorimetric reaction [129] and quickly moved to newer methods involving thin-layer chromatography (TLC) [15,130]. High performance thin layer chromatography (HPTLC) [12,130,131] succeeded TLC, with later on the development of the 4×4 HPTLC, where 4 samples and 4 standards could be developed simultaneously on one HPTLC plate [132-133].
Since the 1960s, separation methods based on GC and LC gained in popularity, due to the achievement of higher specificity and selectivity. With the introduction of GC in thyreostatic drug residue analysis, it became clear that coupling of GC with MS was a requirement to achieve adequate quality control criteria [134-137]. A milestone here was the use of derivatisation agents such as benzylchloride [134], pentafluorobenzylbromide (PFBBr) [135] or methylation prior to the analysis [137-140] resulting in lower limits of detection (LODs). Unfortunately, the recovery of certain thyreostatic compounds, like TAP remained low. Therefore, a more efficient method combining HPTLC and GC-MS analysis was developed [131] with MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide) derivatisation. Furthermore, a second derivatisation step (NBD-Cl [140], PFBBr [141-143] or 3-BrBBr [144]) was implemented and combined with MSTFA derivatisation, tackling recovery, repeatability and active site adsorption issues [144,6].

Finally due to the aroused interest concerning LC, also HPLC methods were described for thyreostat detection in matrices such as urine, meat, serum, plasma and thyroid, combined with UV (ultraviolet) [145-149], electrochemical [137,148], chemiluminescence [150] and diode-array detection (DAD) [151], with only the latter one reaching a LOD of 10 µg kg^{-1} instead of the usual mg kg^{-1} range. With the introduction of API interfaces like ESI and APCI, LC-MS became the method of choice for (thyreostatic) residue control [80,152,153]. The first LC-APCI-MS method was

introduced by Blanchflower *et al.* (1997) [152] without derivatisation. Later on, LC-MS^n and LC-MS/MS methods in combination with ESI were developed and applied on matrices such as urine, faeces, muscle, liver, animal feed and hair, which obtained higher sensitivity and specificity by derivatisation with NBD-Cl [153] or 3-iodobenzyl bromide (3-IBBr) [80] (Figure I.12). Within the advantages of ion trap mass spectrometry, multiple stage mass spectrometry (MS^n) originated for confirmatory purposes [154]. Evolution towards UHPLC-MS/MS methods, due to the high sensitivity, selectivity and thyreostat identification potential, proved derivatisation was no longer necessary for the analysis of thyreostats in urine [83].

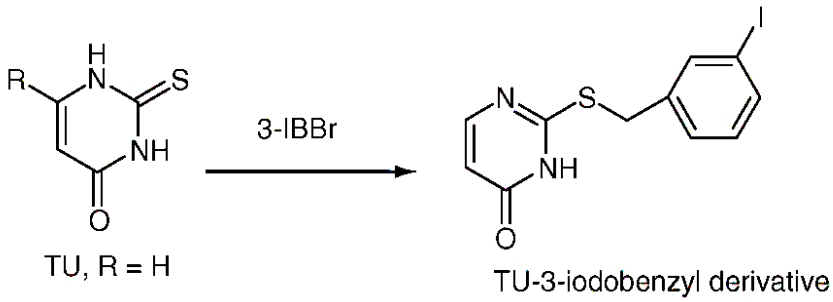


Figure I.12: Derivatisation of thiouracil with 3-iodobenzyl bromide [1].

More recently, detection has evolved towards full scan high resolution mass spectrometry (HRMS) approaches, which includes time-of-flight (e.g. TOF and hybrid TOF (Q-TOF)) and Orbitrap (e.g. single-stage (Orbitrap Exactive) or multiple-stage (LTQ-Orbitrap, hybrid quadrupole Orbitrap (Q-Exactive)) technologies, providing resolutions up to 150,000 full with at half maximum (FWHM) [155,157]. The increased selectivity in comparison with tandem mass spectrometry [157,158], the potential of detecting numerous known and unknown compounds at once, as well as, the possibility of post-acquisition data mining demonstrate the great potential of these techniques for residue analysis as a whole. Not surprisingly, an UHPLC-HESI-HRMS Orbitrap multi-residue method for the analysis of banned veterinary drugs including thyreostats has been described in bovine urine [159].

4.3. Thyreostatic drugs analysis in animal feed

The analysis of animal feed has not been the first priority in the monitoring plan of thyreostats when they were banned in 1981 [20]. Most analysed matrices were of animal origin, including thyroid gland, muscle tissue or urine. One method resorted to micellar electrokinetic chromatography (MEKC) for the detection of thyreostats in animal feed by DAD reaching LODs of 0.4-0.6 µg g^{-1} in fodder and 4% peak area

repeatability [160]. A capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection method was developed in urine achieving an LOD range of 0.30 (TU)-0.43 (PhTU) ng mL^{-1} [161]. Subsequently, it was applied to feed with a mean recovery at 0.6 µg g^{-1} of 78 ± 3% and RSD < 3%, but no LOD specifications in feed were mentioned. Thereupon a similar CE method was developed for animal feed using a different type of detector, an electrochemical detector and adding a solid-phase extraction pretreatment allowing LODs from 7.6-25 µg kg^{-1} (18 ng g^{-1} TU; 25 ng g^{-1} PTU) for various thyreostats [162]. Recently, an ion-pair based hollow fiber liquid phase microextraction (IP-HFLPME) coupled with HPLC-ultraviolet detection (HPLC-UV) method was developed for the analysis of another therapeutic synthetic thyreostat (methimazole) in animal feed reaching an LOD of 0.5 µg kg^{-1} and RSD% for feed of 5.6% [163].

Meanwhile also mass spectrometrical methods were developed preceded by either gas or liquid chromatographic separation. Similarly to other methods, the developed methods were originally intended for other matrices e.g. urine but applied to animal feed. Therefore little to no validation data were available. In this light, HPLC-MS (LCQ ion trap) [153] and LC-MS/MS [69] detection methods with an incorporated derivatisation step were developed. The latter one was the first to try the extraction of thyreostats from Brassicaceae feeds to prove the link between feed and thiouracil detection in urine of livestock. In this study a one-step methanolic solid/liquid extraction, derivatisation, liquid liquid extraction (3-steps) and silica cartridge solid phase purification were followed. Vanden Bussche *et al.* (2011) [164] further optimized the procedure from Pinel *et al.* (2006) [69] for Brassicaceae by amongst others adding a hydrolysis step with the plant-derived enzyme myrosinase showing thiouracil could be detected in many Brassicaceae derivates (i.e. broccoli, cauliflower, rapeseed and coarse colza '00' meal). A recent report used methanol and petroleum ether as extractions solvents without myrosinase followed by derivatisation (3-IBBR) and diethyl ether liquid liquid extraction to analyse thyreostats in animal feedstuff by LC-MS/MS [165]. The validation data obtained in this study were however disappointing, with recoveries for TU ranging from 77.7-86.9% and CC_{α}- CC_{β}-values of respectively, 2.39 and 4.07 µg kg^{-1}.

4.4. Thyroid hormone analysis in a clinical setting

Thyroid hormones can occur either bound to specific thyroid hormone-binding proteins or unbound and be bioactive in the blood circulation. Between both fractions equilibrium is established, therefore detection of thyroid hormones must be able to portrait both since they have an important diagnostic potential

(hyperthyroidism, hypothyroidism). Both fractions require a specific procedure in order to be detected; for free thyroid hormone separation from bound hormone is crucial, whereas total hormone detection needs denaturation to also free the bounded fraction.

4.4.1.Total thyroid hormone analysis

Immunoassays, like RIA and ELISA, have been widely used for detection of unbound/bound thyroid hormones in both, animal (equine, feline, canine etc.) [126,166-168] and human analysis [169-171], of thyroid hormones in blood. These immunoassays are quite comparable, as with ELISA antibodies are labelled with an enzyme (chromogenic, fluorogenic or electrochemiluminescent reporters), and in RIAs with a radioactive label. The use of radioactive isotopes, however, requires appropriate licensing, storage facilities, safety devices, and record keeping, which increase the operating cost. As an alternative, non-radioactive immunoassay methods, such as, chemiluminescent immunoassays (CIA) and a chemiluminescent-enzyme-immunoassays (CEIA) have been developed for the analysis of thyroid hormones in blood samples [172].

Regarding the analysis of the total thyroid hormone fraction, a lot of RIAs are commercially available and commonly accepted as the golden standard in veterinary medicine (e.g.: cats, dogs) [126]. In general solid-phase RIAs are used, in which the antibodies are immobilized to the wall of a polypropylene tube. Next, a 25 μL aliquot of the treatment, standard (supplied with the kit) or control sample is added to the coated tubes. Plain tubes are used for determining the non-specific binding and the total counts. A 100-μl aliquot of ^{125}I-labeled T_4 is added to each tube. The samples are subsequently incubated, decanted and radioactivity is measured for 1 min in a gamma counter. The concentration of T_4 is determined using regression analysis [172].

Kemppainen & Birchfield (2006) [173] have studied the difference between a RIA marketed for use in dogs, a RIA for use in humans, a chemiluminescent enzyme immunoassay for use in humans, and an in-house ELISA for dog and cat serum. The correlation between all 4 methods proved to be good, even though the RIA specifically developed for dogs, consequently came up with lower concentrations in the higher range of the concentration spectrum, predominantly in cats. Several factors were enumerated, which could contribute to this lower T_4 concentration, including: the assay used calibrators of canine origin, the lower calibration range and lower sensitivity of the test [173]. Because this could be important in veterinary diagnostics, adapted reference ranges based on the applied methods capacity

would be useful to adequately evaluate animals and categorize their thyroid illness. Significant biases for immunoassays have been reported due to either endogenous factors (e.g., abnormal binding proteins, dialyzable protein binding competitors, heterophile antibodies, autoantibodies) or *in vitro* factors (free fatty acids, assay antibodies, analogues, intrinsic dilution). Disagreement between results from different methods has been a perplexing problem, limiting their clinical utility [174,175].

Therefore over the years, increasing replacement of immunoassays took place in favour of more sensitive and specific MS techniques (GC-MS; LC-MS; LC-MS/MS) in total and free thyroid hormone applications in humans [176-181]. For total thyroid hormone detection in particular, laborious sample preparations were common with the first GC-MS applications [182,183], but over time evolution towards LC-MS based methods, with eventually HPLC-isotope dilution tandem MS (HPLC-ID-MS/MS) applications, allowing a higher throughput with a reduction of complexity in extraction procedures emerged [184-186]. The principle of ID-MS is surprisingly simple. A sample with known isotopic composition of the analyte element, but unknown elemental content is mixed with a known amount of spike. This spike contains the analyte element in a different isotopic composition, ideally enriched with the rarest natural isotope. After complete mixing of sample and spike the so-called sample-spike blend or isotope-diluted sample gains a new isotope ratio that will lie between the isotope ratio of the sample and that of the spike. This altered isotopic-composition directly reflects the analyte concentration in the sample (Figure I.13). In order to quantify successfully with ID, a minimum of 2 stable radioactive isotopes of T_4 are required for mass spectrometrical analysis. For total thyroid hormone analysis, bound and unbound T_4 are measured, therefore after enriching the serum sample with isotopic-labelled T_4, protein denaturation is applied (e.g. acetone). After denaturation and centrifugation the subsequent supernatant is separated in time based on liquid chromatography and mass spectrometrically analysed [183,187]. ID-MS is a very quick (below 7 min run), accurate (based on ratio not signal intensity), specific (measures only the analyte), precise and easy way to analyze total thyroid hormones [184,187]. Even though, ID-MS, has some disadvantages, these are easily overcome by the overwhelming advantages of the technique. As a disadvantage may count that ID-MS is a destructive method and requires two isotopes, ideally completely free from any isobaric or polyatomic interferences. In case of mono-isotopic elements ID-MS can, therefore, only be applied if a long-lived radionuclide, such as ^{129}I, is available for spiking. The most important issue, however, is the mixture between sample and spike isotopes, since a complete isotopic equilibrium between sample and spike isotopes is required to have satisfying results [187].

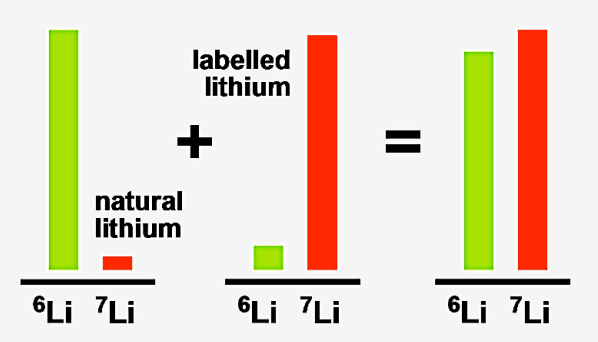


Figure I.13: Principle of isotope dilution described for lithium (^{6}Li and ^{7}Li) (©2014 Wikipedia).

4.4.2. Free thyroid hormone analysis

Free thyroid hormone analysis is regarded as the most sensitive diagnostic test for thyroid disorders. Notwithstanding, the detection of the free fraction of thyroid hormones requires a specific approach for its extraction. The free thyroid hormone fraction should be separated from the bounded fraction without disturbing the physiological equilibrium between both, after which free thyroid hormone concentrations may be measured [188]. To this end, specific separation techniques can be applied: equilibrium dialysis (ED) or ultra filtration (UF) [189]. To attain a representative separation of both free and bounded thyroid hormone fractions, physiological conditions should be respected taking into account: time, temperature, pH, sample dilution and change in ion composition during separation [188]. Since, the free fraction represents ppt-level (pmol L^{-1}) concentrations in serum, whereas total hormones represent ppb-levels (nmol L^{-1}) concentrations, this potentiates its susceptibility to interference even more, in comparison to total hormone detection [184].

Mainly the separation of free thyroid hormones is still traditionally based on equilibrium dialysis (ED) in combination with immunoassays, but lately MS applications, like ED LC-ID-MS/MS have also been described and put forward as a human reference method [170,177,190]. In animals, however free thyroid hormone detection is as yet mostly performed with ED and immunoassay combinations [127,166-168].

The principle of ED is based on the affinity of thyroid hormones for their binding proteins. The dialysis cell contains two compartments separated by a semi-permeable membrane, in which one compartment (donor cell) contains the serum sample and the other compartment (acceptor cell) contains only a protein-free dialysis buffer [186]. Exclusively the unbounded fraction can pass the semi-permeable membrane to the acceptor compartment, based on pore-size (cut-off). The dialysis

cell equilibrates whilst incubating, after which both donor and acceptor cells are analysed to calculate the free T_4 concentration [191].

ED has the disadvantage to be a labor-intensive, imprecise, technically demanding and costly procedure, which is hard to implement in clinical laboratories where the amount of samples analyzed per day prevails [169,192]. A new current possibility allows performing ED batch wise using dialysis in 96-well plates [170]. Problems reported with ED are also related to the dilution and dialysis buffer composition [177]. Because of all those setbacks, UF was proposed as a valuable alternative for ED [179,190,193], which further led to its implementation in several North American laboratories for human serum analysis nowadays [169].

UF consists of centrifugation of undiluted serum, through a specific disposable UF centrifugal device mountable in the rotor of a centrifuge with a specific cut-off semi-permeable membrane, which takes only around 30 min whereas ED takes around 24 h [169,170,192,194]. Centrifugal force causes the unbound hormone fraction to pass through the membrane, therefore the conditions (37 °C, neutral pH) of centrifugation should closely relate to the *in vivo* circumstances in order to have a physiologically accurate view of the *in vivo* status of thyroid hormones [194]. Pre-washing of the UF filters (phosphate buffer-ultrapure water) might be called for in order to exclude potential interfering compounds (e.g.: glycerine) [195]. The major concerns with UF include: non-equilibrium, absorption, protein leakage, protein binding, wall adsorption and temperature control [177,194]. For example a leakage of 0.005% of serum protein into the ultrafiltrate will already cause an increase in concentration of free T_4 [195]. Therefore, it is preferable that the analysis of the samples swiftly follows the separation and extraction procedures either through RIA or even better with mass spectrometrical techniques [194]. For free thyroid hormone detection in serum ID-LC-MS/MS is a sufficiently sensitive, specific, accurate and precise method of analysis when accurately executed, therefore it has been put forward as a potential reference detection method in humans [170,183,193].

Finally, notwithstanding the excellent performance of LC-MS/MS analysis in both free and total thyroid hormone analysis compared to immunoassays, it has no in-house potential. Medical practices (animal or human) cannot execute these analyses themselves unless they dispose of an elaborate laboratory facility, which is often not the case and makes the results not directly available for further diagnostics [169].

5. INTESTINAL BACTERIA IN LIVESTOCK

5.1. Gastro-intestinal tract of monogastric and polygastric animals

The GI tract of animals can be divided into two subgroups depending on the anatomical segmentation of their stomach, respectively called mono- (one segment, e.g. pig) and polygastric animals (many segments, e.g. cow). Besides, another well-known subdivision according to the animals feeding behaviour identifies three subgroups: carnivorous, omnivorous and herbivorous animals. The overall GI morphology in animals is greatly influenced by adaptation, nature and, frequency of food intake, storage need, body size and body shape [196].

Basically, the GI tract is a long chain of subsequent chambers, which in the end results in degraded feed, and resorbation of a battery of nutrients essential for the animal. A first step in this process takes place in the oral cavity where feed is grounded and homogenized with saliva with the help of a good set of teeth, which explicitly include grinding premolars and molars in herbivores. Also the process of ensalivation helps digestion and bolus formation, since saliva mainly contains mucin, although in pigs also starch-degrading amylase is present. Per day cows can produce around 200 L of saliva, which plays a role in sustaining the nearly neutral pH (5.5-6,5) of the rumen, whereas men only produce 1-2 L. In cows the tongue has filliform papillae on its surface with a highly cornified epithelial layer, creating a rough surface. After bolus formation in the mouth the feed is swallowed moving through the pharynx into the oesophagus and stomach. The monogastric stomach (Figure I.14-B) consists of one sac divided into different regions (Figure I.14-C). The bolus enters the stomach through the cardia sphincter into the non-glandular oesophageal region. The subsequent areas of the stomach are cardiac-gland region, which produces mucin to protect the stomach epithelia from chemical and mechanical deterioration, the fundic gland region produces besides mucin (neck cells) also pepsinogen (chief cells) and HCl (parietal cells) which converts pepsinogen to pepsin acting on proteins in the stomach. Subsequently, the feed bolus moves through the pyloric region where G-cells in the pyloric glands can produce gastrin that also stimulates HCl and pepsin production. The pyloric sphincter at the end controls the outgoing throughput of the stomach [197].

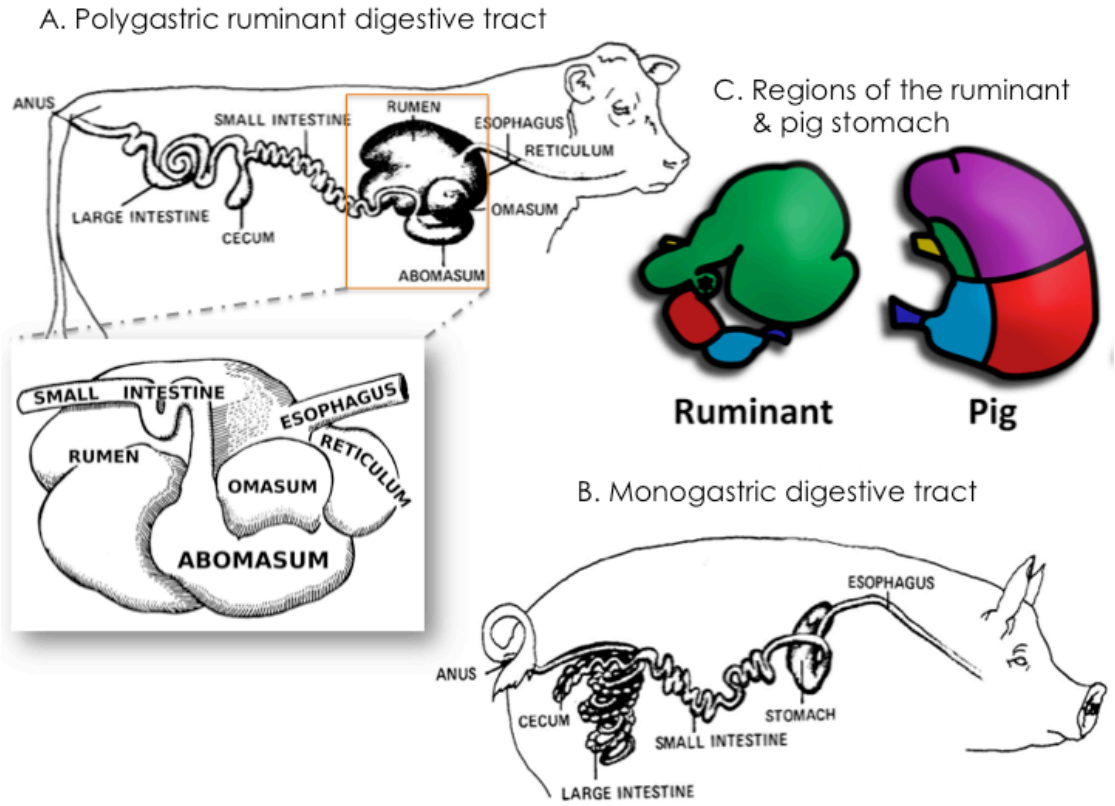


Fig I.14 : Overview of a polygastric (ruminant) (A) and monogastric (pig) (B) digestive tracts. The regions (C) are represented with the following colours: yellow: oesophagus; green: glandular epithelium; purple: cardiac glands; red: gastric glands; blue: pyloric glands; dark blue: duodenum (©2014 Wikipedia and ©2012 Texas A&M University).

In the polygastric animal four subsequent sacs act as stomach: rumen, reticulum, omasum and abomasum (Figure I.14-A). In general the oesophageal region of monogastric animals compares to the rumen, reticulum and omasum whereas the abomasum can be compared to the gland regions. Due to these three forestomachs or proventriculi, ruminants are able to utilize cellulose present in plant material. The ruminal environment is prone to growth of microbiota (bacteria, protozoa, fungi), which form cellulase and convert plant material into volatile fatty acids, CO_2, ammonia and synthetize vitamin K and B complex vitamins. In the end even the microbial cells are digested. Non-protein nitrogens like ammonia and urea can be reincorporated in the microbial protoplasm and redigested by the organism into valuable amino acids. The rumen has also an oesophageal groove which when closed can bypass the rumen from the oesophagus directly to the omasum. In young milk-drinking calves this is an important feature that prevents acidosis of the rumen, allowing coagulation of the milk by renin in the stomach before further digestion. In

the rumen three phases are present: the lower liquid partially digested food, the middle layer with not yet entirely soaked food and the upper layer consisting of a gas phase (CO_2, methane). The muscular sac contracts regularly mixing different layers together and when a high-fiber bolus blocks the cardia this will be regurgitated for further mastication. When the methane concentration increases above a certain level this is ventilated through the oesophagus and called ructus. Once feed particles are fine enough they pass through the reticulums' honeycomb structure to the omasum or bookstomach. There, muscular laminae studded with short blunt papillae descending from the dorsal portion of the omasum induce a grounding motion on the feed bolus before entering the abomasum or 'real' stomach. The omasum to the abomasum changes abruptly into a mucus producing epithelium, with consecutively more or less the same gland regions as in single stomach animals.

Finally the intestines are reached, first the small (duodenum, jejunum ileum) where bile (bile salt, cholesterol, lecithin, bile pigments) and pancreatic (sodium bicarbonate and enzymes (o.a.: amylase, lipase, trypsin)) juices indulge on the feed chime, degrading proteins, fats and carbohydrates. Various hormones and the presence of degradation products regulate all GI processes. In both the pig and cow the ileum terminates into the caecum and subsequently into the colon or large intestine (ascendens, transversum and descendens). In pigs (Figure I.14-B) the topography of the colon is coiled resulting in a typical cone-shaped appearance. The microbial population in the large intestine can degrade the cellulose present in the chime of high roughage single stomach animals, like horses. Since pigs are more omnivorous the size of the colon is more restricted than in horses and even more so in cows as they are fore-stomach fermenters. The rectum and anus with a sphincter make up the end of the GI tract [197].

5.2. Intestinal microbial populations in livestock

The GI tract contains a complex and diverse population of microorganisms, which for most animals have not yet entirely been elucidated [198]. The distribution of microorganisms over the digestive tract depends on the type of fermenter, either forestomach fermenters (cow) with a fermentation chamber prior to the acid-secreting part of the stomach, or hindgut fermenters, which use the caecum and colon as microbial fermentation chambers (pig). Herbivores can be both forestomach (cow) and hindgut fermenters (horse) [199].

In porcines the Gram-positive anaerobical organisms prevail in the intestine (e.g.

facultative anaerobic *Streptococcus sp., Eubacteria sp., Clostridium sp., Lactobacillus sp., Peptostreptococcus sp.* etc). If Gram-negative bacteria are encountered they are mostly *Bacteroides sp.* [200-202]. Pigs have been reported to posses in their hindgut: *Streptococcus sp., Lactobacillus sp. (e.g. L. acidophilus), Eubacterium sp., Fusobacterium sp., Bacteroides sp. (B. multiacidus), Peptococcus sp., Peptostreptococcus (e.g. P. elsdenii), Streptococcus sp., Bifidobacterium sp. (e.g. B. suis, B. longum ssp. suis, B. pseudolongum ssp. pseudolongum and ssp. globosum, B. thermophilum, B. boum, B. choerinum), Selenomonas sp., Clostridium sp. (e.g. Cl. perfringens), Butyrivibrio sp., Escherichia sp. (e.g. E. coli), Prevotella sp., Ruminococcus sp., Veillonella sp., Leptotrichia sp., Anaerovibrio sp., Gemminger* sp., *Leptotrichia sp.* [200,203-205].

Bovines have microbial activity concentrated in the rumen and the large intestine. The rumen contains obligate anaerobic bacteria and archaea but also fungi and protozoa [199,206]. Primary ruminal bacteria that break down nutrient from feed are carbohydrate degrading bacteria (amylolytic or cellulolytic) and proteolytic bacteria [206]. Secondary ruminal bacteria use degradation products from the primary bacteria for their metabolism, like methane producing and lactic acid degrading bacteria [207]. The constitution of the ruminal but also colonic microbial populations can be altered under the influence of diet, age, health, geographical location and seasonal changes [206,208]. Therefore, these variations also influence the quantity and nature of the fermentative end products [199]. In the rumen *Bacteroides sp., Eubacterium sp., Peptostreptococcus sp*. can also be found similar to those in the colon. More specific for the rumen are *Ruminococcus sp., Butyrivirbrio sp., Streptococococcus sp.,* methano-bacteria (*Methanobacterium sp.*) [199]. *Megasphaera sp., Butyrivibrio sp., Ruminobacter sp., Cytophaga sp., Roseburia sp.* and *Selenomonas sp.* were reported exclusively in ruminal fluid according to Callaway *et al.* (2010) [208]. Most protozoa in the rumen belong to the ciliated type of the holotrichs, which rapidly absorb sugars and also store sugars as starch within their structure (reserve) and the entodiniomorphs, which feed on cellulose and bacteria. Some species are also carnivorous on other protozoa [199]. Hindgut bacteria in cattle mainly include *Clostridium sp. (Cl., butyricum), Bacteroides sp., Porpyhyromonas sp., Ruminococcus sp., Alistipes sp., Succinivibrio sp., Lachnospiraceae, Prevotella, Lachnospira sp., Enterococcus sp., Oscillospora sp., Cytophage sp., Anaerotruncus sp., Acidaminococcus ssp., Bacillus sp., Streptococcus (S. bovis, S. faecium), Micrococcus sp., Escherichia sp. (E. coli, E. freundii), Klebsiella sp., Proteus sp., Streptomyces sp., Candida krusei* (fungi), *Mucor sp*. (fungi) [208-210].

5.3. *In vitro* modelling of the intestinal tract

The GI tract can be studied, either through *in vivo* (live animal), or *in vitro* (simulation of the GI) experimental research. Using *in vivo* studies, information on the impact of the GI processes is gathered through sampling of the various compartments of the GI tract (stomach, small intestine, large intestine) and feces. In practice this is technically quite complicated, and in many cases fistulation of the animals is required, which raises ethical questions towards animal welfare. Therefore such trials are closely regulated and require ethical committee agreements. Overall an estimated twelve million animals per year are used for experimentation according to the EU Strategy for Protection and Welfare of animals 2012–2015. Although this is still a lot, nowadays, simplifying animal experiments, reducing discomfort, setting human endpoints and most importantly if possible electing an alternative *in vitro* experimental set-up are widely promoted. Beside, the cost of animals, housing, trained personnel etc. is such that setting up a series of *in vivo* studies, is just not feasible [211].

The GI tract can be imitated by using *in vitro* simulations and with this the invasive character and need for animals vanishes, which makes the procedure more cost-effective. Moreover the amount or time of sampling is no longer an issue making it the more responsible choice for feed/food digestion experiments [212] Digestion simulations can be subdivided in dynamic and static *in vitro* set-ups [213].

The dynamic digestion simulation is constituted out of different reaction jars that recreate a specific phase of the GI tract (stomach, small intestine or large intestine). The subsequent reaction tanks are interconnected and reaction products can be removed or not through absorption [211]. The first developments focussed on specific compartments of the GI tract, like Miller & Wolin (1981) [214] who proposed a semi-continuous culture system, followed by Veilleux & Rowland (1981) [215] introducing a two phase continuous culturing system and Gibson *et al.* (1988) [216] which used a three-phase continuous culturing system.

The need for partially or fully integrated *in vitro* systems, which mimicked the gradual transit of ingested compounds through the digestive tract, grew increasingly over the years [217-219]. Currently, a well-known configuration is Simulator of the Human Intestinal Microbial Ecosystem (SHIME) (Figure I.15) [217]. The SHIME has 5 tank reactors being: stomach (1), duodenum, jejunum, ileum (2), caecum and colon ascendens (3), colon transversum (4) and colon descendens (5), which are all kept at 37 °C.

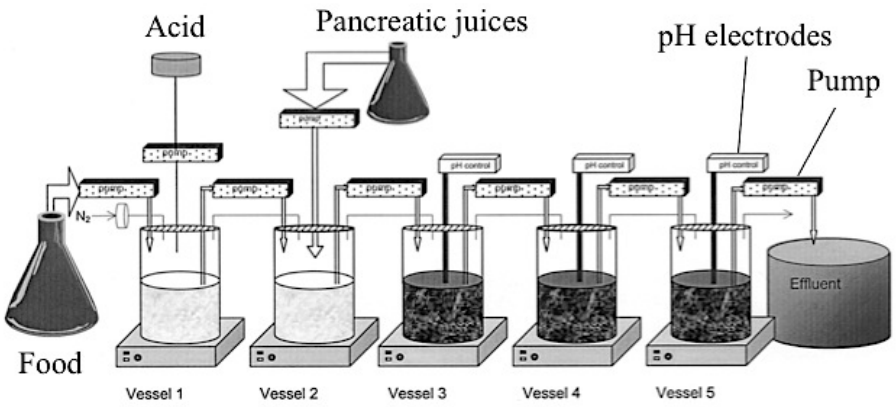


Figure I.15: Scheme of the Simulator of the Human Intestinal Microbial Ecosystem set-up [220].

The first two vessels (stomach and small intestine) work according to a fill-and-draw system, whereas the last three (large intestine compartments) are continuously stirred tank reactors with a total retention time of 76h. The suspensions are added with the necessary fluids for the specific compartment (e.g. HCl (stomach (1)), bicarbonate, pancreatic and bile juices (small intestine (2)), intestinal bacterial inoculum (large intestine (3-5)) and their pH continuously monitored and adjusted when necessary. Therefore, the SHIME model is recognised as a good *in vitro* simulator of the human GI in *vivo* process, which for static digestion simulations is less the case [221].

Similar dynamic digestion models have been developed for livestock, pigs and cattle in order to evaluate amongst others their feed conversion and bioavailability with respect to certain feeds. A dynamic digestion model was developed, simulating the gastric digestion of pigs [222]. This model was constructed to include the chemical and physical changes associated with gastric digestion, such as, enzyme release, digestion product removal and gastric emptying. A variation of the latter set-up was reported by Rivest *et al.* (2000) [223], which developed a system to specifically evaluate protein degradation in the small intestine of pigs. The main characteristics of the model were the following: the small intestine is divided into several segments of variable length but with equal digesta retention time; the rate of transfer of digesta between segments is based on the progression of myoelectric migration complexes; pancreatic and biliary secretions are poured into the first segment, whereas intestinal secretions enter all intestinal segments; protein hydrolysis is described by first-order equations; and an intestinal absorption capacity is used to estimate absorption of hydrolysed protein. This model is useful in the evaluation of feeds and feeding strategies. A more elaborate dynamic model had already been developed

consisting out of stomach, duodenum and proximal jejunum, distal jejunum, ileum and large intestine by Bastianelli *et al.* (1996) [224]. In each of these anatomical compartments, subcompartments correspond to the major biochemical components of feed (e.g., fats, proteins) and their products of degradation (e.g. amino acids; fatty acids). The major degradation and absorption events are considered as well as the effect of microbial activity in the large intestine. The total number of compartments is 44 [224].

In bovines the main concern in feed digestion is the ruminal digestion, which is also reflected in the amount of publications concerning ruminal evaluations of feeds and additives based on static and dynamic *in vitro* digestions [220-233]. A broadly applied [230,237-239] dynamic *in vitro* ruminal system is the RUSITEC system (Figure I.16) that was developed by Czerkawski & Brekenridge (1977) [240] consisting of four vessels in which the normal rumen microbial population is sustained under strict controlled conditions over time [241].

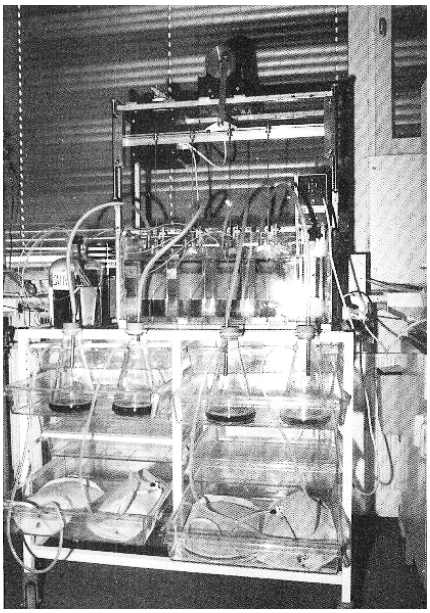


Figure I.16: The early Rusitec system: *in vitro* dynamic rumen simulation (©ikasbil.net).

Furthermore, Gérard-Champod *et al*. (2010) [212] optimized an intestinal system to evaluate the effect of certain additives on digestion in veal calves. The final system was based on a continuous culture system comprising one glass vessel with an operating volume constantly maintained by a level probe controlling the outflow of the fermentative content. A sterile nutritive medium was introduced continuously at a rate of 15 mL h^{-1} (retention time, 80 h). The fermentative medium was permanently stirred, the pH was kept at a constant 6.5, and the temperature was maintained at

38.5 °C under anaerobic conditions. This set-up showed good *in vitro in vivo* correlation.

Besides also mathematical models have been described e.g. by Dijkstra *et al.* (1992) [242] simulating the digestion, absorption and outflow of nutrients in the rumen. The model consists of 17 ruminal state variables, representing nitrogen, carbohydrate, lipid, and microbial and volatile fatty acid pools. Based on nutrient information of feed the digestion could be simulated.

In comparison static *in vitro* digestion simulations consist of only one reaction tank, from which the digestion products are not removed. This simulates transit through the digestive tract by sequential exposure of the substrate (food/feed) to simulated digestion conditions (e.g. stomach, small intestine, large intestine) [219]. These models are therefore less comparable with the *in vivo* dynamics, as dynamic *in vitro* simulations are. Notwithstanding, static *in vitro* digestion simulations are preferred when evaluating one phase of the digestion process (e.g. large intestine) or when simple food or isolated nutrients are assessed [211]. Moreover this type of model is simple and cheap, because it does not require a lot of specialised equipment. In general, a static system consists of homogenising feed, adding the adequate medium, adding digestion enzymes or microorganisms, stirring and adjusting pH when needed [211]. Single stage digestions have been described for pigs when evaluating glucosinolate degradation in separate small intestinal and caecal jars [243]. An example of a multi-stage static digestion model is the *in vitro* simulation of three subsequent enzymatic digestion steps: stomach, small intestine, large intestine in one reaction tank [244,245]. The digestion protocol of Boison & Fernandez (1997) [244], which has been specifically developed for porcine simulation, was used as an example in this study. With respect to the ruminal *in vitro* static digestion, it merely consists of freshly collected ruminal fluid that will then be filtered, buffered and conveyed into a flask for incubation (39 °C) after CO_2 flushing. Many useful experiments have been conducted based on this general set-up adapting incubation time, sampling, feed type, etc [229,231-234,246,247].

5.4. Bacteria with myrosinase-like enzymatic activity

Bacteria may own several enzymatic activities that are either permanent or adaptive to the gut environment [248]. These enzymes may be classified into different groups: oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase [249]. One enzymatic activity, which showed in particular interesting in light of this work, and has been reported for several microorganisms (bacteria and fungi) involves their

ability to degrade GLs through the effect of a myrosinase-like enzyme (Table I.4). The latter bacterial factor would exert a similar effect as the plant-derived myrosinase, thereby inducing the formation of similar degradation products. The mechanism of bacterial myrosinase has been shown to potentially rely on a cell-dependent activity [41,43]. Palop *et al.* (1995) [41] described that myrosinase activity in *Lactobacillus agilis* was cell-dependent since in the absence of intact bacterial cell degradation of sinigrin did not occur. Cheng *et al.* (2004) [43] reported similarly that in cell-free supernatants of various *Bifidobacteria sp.* hydrolysis of sinigrin could not take place and added the fact that after sonication of cells hydrolysis did occur. This demonstrates intact cells were not prerequisited for myrosinase like activity.

Since hydrolysis of GLs by plant-derived myrosinase elicits formation of certain natural goitrogenic substances, the hypothesis postulated that the same could occur in the intestine of animals for synthetic goitrogens. However, before potential parallels with plant-derived myrosinase can be made, this bacterial myrosinase activity needs further characterization of its mechanism.

Table I.4: Overview of the reported microorganisms in literature with a glucosinolate degrading activity.

Micro-organism with myrosinase activity	Origin	Glucosinolate source	Reference
Aspergillus niger AKU 3322	-	Sinigrin	[250]
Apergillus clavatus (II-9)	-	Sinigrin, sinalbin	[251]
Fusarum oxysporum			
Aspergillus sp. NR-4201	-	Sinigrin	[252]
		Brown mustard seed meal	
Geotrichum candidum	-	Rapeseed, only partial goitrin degradation	[253]
Trichosporum cutaneum	-	Rapeseed meal	[254]
Bacillus cereus		(glucosinolates, vinyl-oxazolidine-thiones)	
Sphingobacterium sp. OTG1	-	sinigrin	[255]
Bacteroides theitaiotamicron (II-8)	Human	sinigrin	[42]
Bacteroides vulgatus (BV8H1)	Human	Rapeseed (meal, '00'-variant without peal)	[40]
Bifidobacterium pseudocatenulatum (JCM 7040)	Human	Sinigrin, glucotropaelin	[43]
Bifidobacterium longum (JCM 7050)			
Bifidobacterium adolescentis (JCM 7045)			
Enterobacter cloacae (no 506)	-	-	[256]
Enterococcus ssp.	Human	-	[257,258]
Enterococcus faecalis	Human	-	[259]
Enterococcus faecium			
Paracolobactrum coliforme X3	-	Sinigrin	[256]
Escherichia coli	-	Progoitrin	[260]
Paracolobactrum aerogenoides			
Escherichia coli (EM0)	Human	Rapeseed (meal, '00'-variant without peal)	[40]
Lactobacillus ssp.	Human	-	[258]
Lactobacillus (LEM 220)	Rat, chicken	Rapeseed	[261,262]
Lactobacillus agilis (R16)		Sinigrin	[41]
Peptostreptococcus spp.	Human	Progoitrin, sinigrin	[257]
Proteus ssp.	Human	Progoitrin, sinigrin	[257]

5.5. Isolation and identification of intestinal bacteria

The isolation and identification of bacteria can be a daunting task, but over the years more specific, selective and reliable methods have been developed. In this study, we specifically focused on the identification of fecal/intestinal bacteria [263-265].

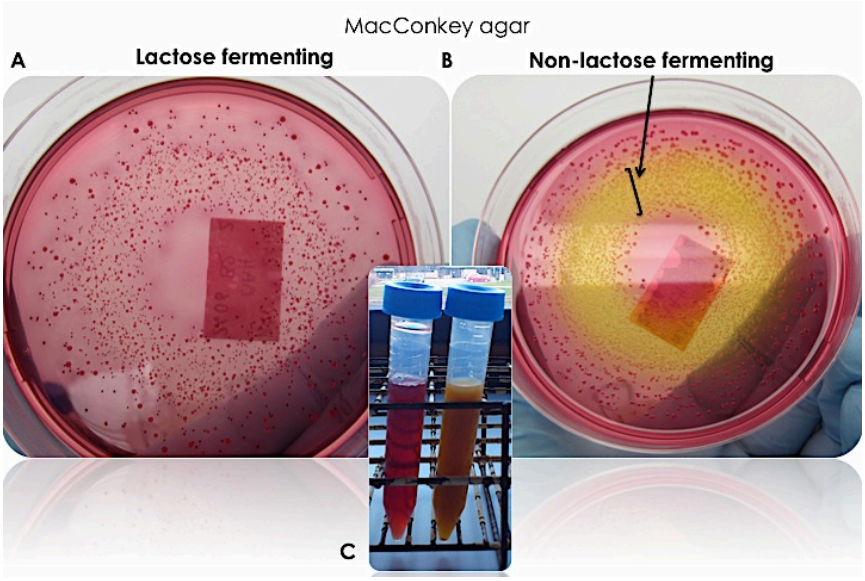


Figure I.17: Lactose fermentation on MacConkey agar (A-B) and in broth (C) from *in vitro* digestion simulation samples. Red-pink colonies are lactose fermenting and colourless-yellow indicates non-lactose fermenting colonies.

The identification of bacteria generally starts with culturing of the unknown intestinal isolates. First, this will be done on a non-selective medium, like Brain heart infusion or plate count agar [265] to evaluate total growth and to enumerate the bacteria aerobically and anaerobically. Enumeration of specific bacterial genera can be achieved on selective media, like Man Rogosa Sharpe (MRS) for *Lactobacillus* and MacConkey (MacC) agar for *Enterobacteriaceae* [265]. Sometimes indication for a certain bacterial species can be achieved by the colour of colonies on a specific differential agar, like MacC, which is both selective, and differential. The bile salts and crystal violet within this agar inhibit growth of Gram-positive bacteria, whereas the lactose can differentiate fermenting (red-pink) and non-fermenting (colourless-yellow) Gram-negative bacteria following the reaction of neutral red to pH alterations (Figure I.17).

Nevertheless, culturing techniques do have some inherent shortcomings including the fact that they select only cultivable bacteria distorting the image of the whole flora and the fact that selective media are not entirely selective and may be toxic for strains within the same genus [265]. Moreover, all culturing techniques fail at

cultivating bacteria that are in a non-cultivable physiologic condition. Despite all these shortcomings culturing techniques remain powerful tools in addition to others [265].

Next, culture-independent techniques including microscopic analysis, enzyme and metabolite analysis can be applied. Microscopy can result in enumeration, evaluation of overall morphology and characterization of isolates (e.g. Gram staining). The detection of certain enzymes (e.g. β-glucuronidase) or metabolites (short chain volatile fatty acids) is an indirect parameter, which represents the metabolic activity of a specific group of microorganisms. Besides, biochemical evaluation of bacterial isolates can be conducted e.g. oxidative/fermentation glucose test (OF) or API kit. The API (analytical profile index) test is widely used in the *Enterobacteriaceae* biochemical identification [266,267]. This is a quick and easy biochemical miniaturized kit, which consists of 20 or more cupules containing dehydrated substrate. When bacterial suspensions are added and incubated at 37 °C for 18-24 h the reaction causes a specific colour change in each well. Every colour reaction stands for a numerical classification, which in the end results in a numeric code when all wells are considered. This code will then be matched against the APIweb™ database in order to identify the isolate with a percentual certainty. The API 20E is considered one of the 'gold standard' methods for biochemical identification of enteric and Gram-negative bacteria in clinical and food laboratories [268]. Also belonging to the phenotypical identification procedures for bacterial identification are a.o. serotyping (surface antigen detection e.g. via ELISA), biotyping (based on biochemical and physiological properties), bacteriocin typing (bacterial proteolytic enzymes), phage typing (phages are bacterial viruses specific for particular strains) and antimicrobial susceptibility patterns [249,263-265]. All these phenotypical characteristics are not variable enough for differentiation between closely related strains [263]. Moreover, these are based on the identification of products of gene expression, which in turn are highly susceptible to variations in environmental conditions like temperature, growth phase and spontaneous mutation [264].

Therefore, this has led to the development of more direct identification methods, called genotyping. This procedure achieves determination between strains based on their genetic content. Genotyping had increasing success due to its high resolution and the uniqueness of the fingerprinting.

Techniques for identification and genotyping can be divided into three main categories:

1. DNA banding pattern-based methods
2. DNA sequencing-based methods
3. DNA hybridization-based methods

The discriminatory ability of all these methods except for DNA sequencing is species dependent [263].

DNA banding pattern based methods discriminate strains based on the size of DNA bands (fragments) generated by amplification of genomic DNA or by cleavage of DNA using restriction enzymes. Amplification of DNA creates millions of copies of a genomic fragment, whereas restriction enzymes precisely recognize and cut target DNA at a defined sequence, sometimes both are applied [263]. Separation is done with electrophoresis (Figure I.18) where DNA fragments under the impulse of an electric field are travelling through a gel (e.g. agarose) separating them according to size and charge creating a band pattern after dying the gel e.g. with ethidium bromide. Also Southern blotting can be used, which combines electrophoresis-separated DNA fragments with a filter membrane and subsequent fragment detection by probe hybridization with labelled probes [269].

Amplified fragment length polymorphism (AFLP) by De Vos *et al.* (1995) [270] is a technique, which combines both amplification and enzymatic restriction cleavage. First digestion of genomic DNA is performed with two restriction enzymes (usually MseI & EcoRI) following ligation of the restriction fragments with end-specific adaptors, whereupon PCR (Polymerase Chain Reaction) amplification is performed. Detection of the fragments can be performed by gel electrophoresis, automated DNA sequencers, like in fluorescent AFLP (FAFLP) where amplification is performed with fluorescently labelled primers, which can then be easily read. The advantages of AFLP include: the flexible amount of loci that can be amplified in one PCR reaction, the lower amount of DNA that is needed because multiple bands are derived from the entire genome, no prior knowledge is needed to use this system, good reproducibility (comparable to Pulse-Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism RFLP and better then Multilocus Sequence Typing (MLST)) and discriminating capacity, cost-effective and automation is possible. The disadvantages are the massive amount of information that is produced which requires automation and the fact that the DNA template should be pure and not a mixture of DNAs.

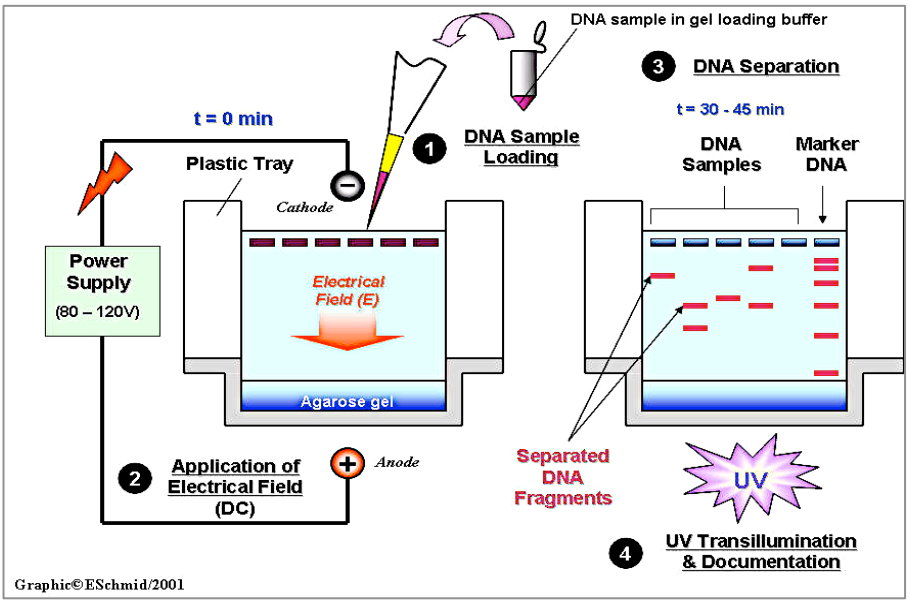


Figure I.18: Simple gel electrophoresis separation of DNA fragments, which after staining can be visualized under UV light (©2001 E Schmid).

DNA sequencing relies on generating the original sequence of the nucleotides and discriminate strains directly based on the polymorphism within their DNA, giving it a high reproducibility. DNA sequencing is very nice when assessing differences and evolutionary changes in DNA (e.g. deletions, insertions, genes under positive selection like antibiotic resistance, virulence) [263,271]. Besides DNA, also ribosomal DNA (rDNA) or rRNA genes are commonly used for sequencing since these are essential for bacterial survival due to its involvement in protein synthesis. The 16S rRNA gene contains 974 variable bases for the domain *Bacteria*. Another gene, 23S rRNA gene contains even twice as many variable bases and is sometimes used as an additional marker in phylogeny analysis. Overall 16S rRNA is the most widely used target gene for identification, because it is highly conserved among bacteria. Therefore the amplification and sequencing of the 16S rRNA gene is still commonly used for identification and phylogenetic classification of prokaryotic species, genera and families. However at the strain level 16S rRNA gene is too conserved, owing to its low evolutionary rate, to be useful, and in particular among *Enterobacteriaceae,* its sensitivity and discriminating power has been questioned [272-276]. Moderately divergent populations can easily be distinguished based on S16 rRNA but with very closely related bacteria other means should be considered. It has been demonstrated that protein-coding gene sequences should be preferred for identification [277]. For Enterobacteriaceae for example *gyr*B (encodes part of DNA gyrase), 16S-23S intergenic spacer, *inf*B gene (encodes an translation initiation

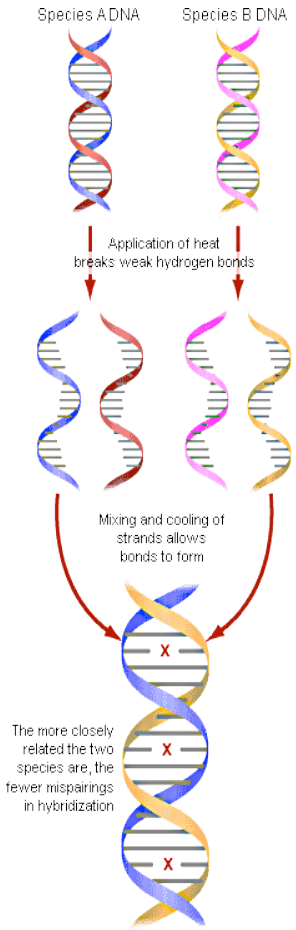


Figure I.19: DNA-DNA hybridization (©2006 University of California Museum of Paleontology).

factor), ATPase β-subunit, the elongation factor Tu and *rpoB* have been described [276,278,279]. In this study *rpoB* gene sequencing was performed since it encodes for the universal bacterial RNA polymerase β-subunit and it is a recognized identification tool [278]. Reports show an evolution towards a combined approach that takes two or more unlinked phylogenetic markers into account for classification of bacteria to improve the understanding of the diversity and complexity of bacterial ecosystems [276,279]. Finally entire genomes can also be sequenced which provides an unequivocal differentiation between organisms, but this is not feasible in a clinical setting, due to its low time and cost-effectiveness compared to other available partial genome methods [263,264].

With DNA hybridization-based methods (Figure I.19) (DNA macroarray or microarray studies), bacterial strains are discriminated by analyzing the degree of hybridization of their DNA to probes of known DNA sequences. When hybridization is > 70% the isolate can be classified as belong to the same species as the probe [280]. Combining this method with rpo*B* gene sequencing has been described in Enterobacteriaceae to delineate bacterial species and genus [280].

Finally, HRMS analysis has lately also been introduced and shown applicability in bacterial identification [281-284]. Mostly used is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). For this technique bacteria are embedded in a matrix whereupon a laser is unleashed, vaporizing the microorganism together with the matrix, leading to ionization of ribosomal proteins. These are the most valuable of all proteins, peptides or nucleic acids, because they are less influenced by culture conditions [283,284]. Subsequently, an electromagnetic field accelerates the ions before entering the flight tube. Each ion traverses the tube at a speed dependent of their molecular weight and degree of ionization, resulting in a specific time-of-flight for each ion. Finally, a specific chromatogram is constructed based on the TOF information, which is unique for a given species and can be matched with database spectra [284].

6. STUDY CONCEPT, AIMS AND OUTLINE

In this thesis the elucidation of the mechanism of the natural occurrence of thiouracil (TU) in livestock was addressed, since this component is a EU-banned substance of primarily synthetic origin. At the time, certain secondary plant metabolites, called glucosinolates, were known to cause the natural formation of thyreostatic components upon plant disruption through myrosinase hydrolysis, a plant-derived enzyme. This suggested that glucosinolate-rich crops, predominantly from the Brassicaceae family could, given the right conditions, potentially form TU precursor(s). Subsequently, the fact that certain human intestinal bacteria had been reported to possess a myrosinase-like activity or the potential to degrade glucosinolates strengthened this hypothesis.
Besides, TU had already been detected in bovine urine upon administration of Brassicaceae feedstuff [69] and the analysis of TU in animal feeds showed TU could be isolated from certain Brassicaceae feedstuffs upon myrosinase addition [160].

Figure I.1 resumes the concept of the present study, which conveys into the following aims:

Evaluate the level of involvement of intestinal microbiota in thiouracil formation upon Brassicaceae *in vitro* digestion simulations in livestock.

Prospect the influence of various glucosinolate-rich crops depending on their origin and pre-treatments in TU formation upon *in vitro* digestion in livestock.

Elucidate the mechanism bringing about thiouracil formation in livestock taking into account various influential parameters.

Develop and validate a routinely applicable extraction procedure for mass spectrometrical analysis of TU (LC-MS^2) from various animal feeds.

Develop and validate an UHPLC-MS/MS detection method for thyroid hormones from bovine serum to allow in a later phase to evaluate the biological effects of endogenous versus exogenous TU.

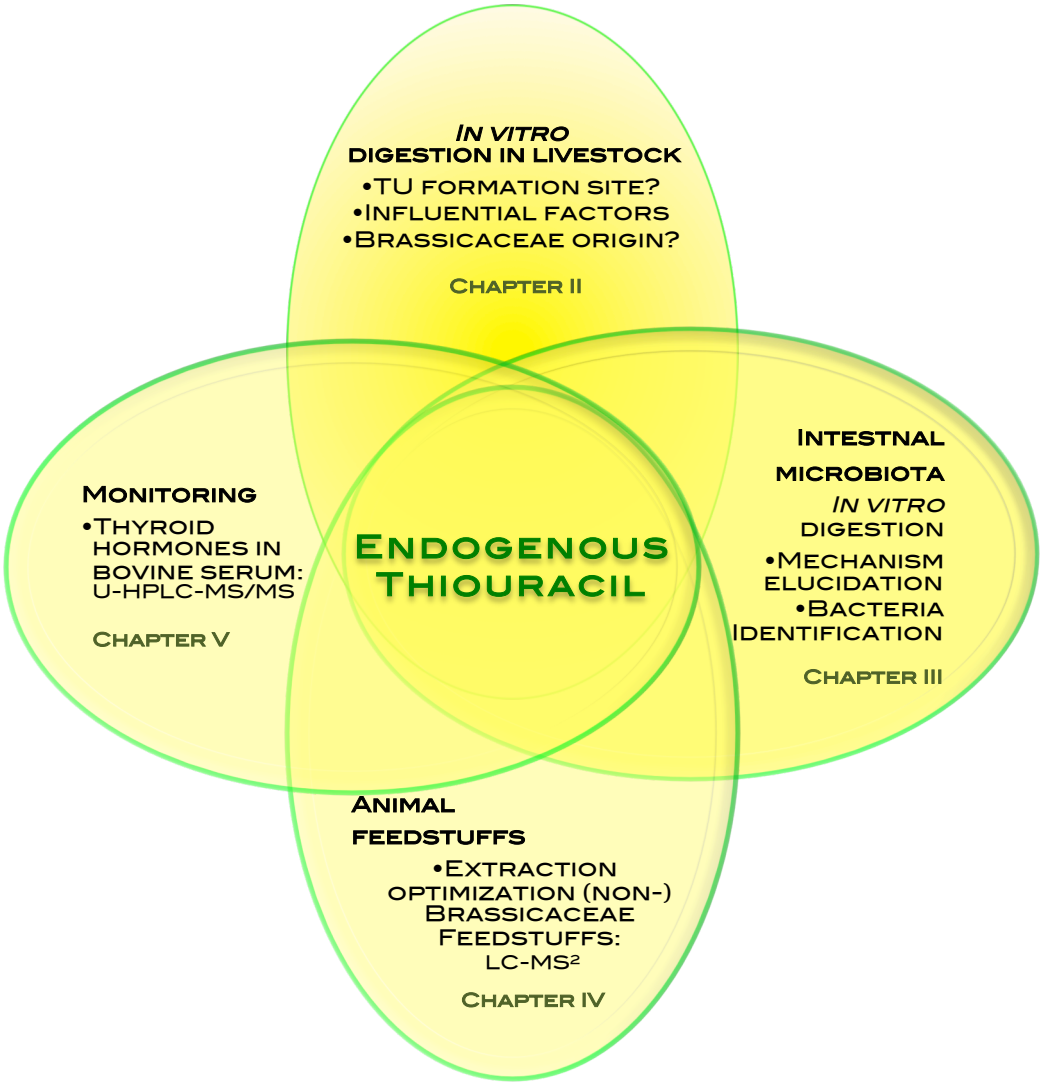


Figure I.20: Crucifer-like shaped graph representing the aims of this doctoral thesis.

Next to **CHAPTER I**, which covered the general introduction on thyreostatic compounds in all their facets, this work contains five additional chapters.

In **CHAPTER II** the possibility of endogenous TU formation by bacterial influence is investigated through *in vitro* digestion in bovines and porcines with various Brassicaceae foods and feeds (rapeseed, coarse colza '00' meal, broccoli, cauliflower).

Subsequently, in **CHAPTER III** the occurrence of endogenous TU formation will be further explored with various pre-treatments of the porcine faecal inoculum upon Brassicaceae *in vitro* simulations and identification of TU forming bacteria is envisaged.

In **CHAPTER IV** extraction optimisation and validation of a mass spectrometrical approach for the detection of TU from various animal feeds is reported, since this remains a crucial control point to asses whether TU is illicitly administered or naturally formed.

Then in **CHAPTER V** the development and validation of a thyroid hormone detection method in bovine serum for free and total thyroid hormones, is described. This method allows to investigate the physiological status of the thyroid gland in livestock upon Brassicaceae digestion, thereby gaining more insight in the relation between thyroid hormones and endogenous feed-related TU production or illicit thiouracil administration.

Finally, in **CHAPTER VI** the general discussion will distil the main conclusions and future perspectives will be addressed.

7. REFERENCES

[1] **Boison, J. 2012.** Analysis of antithyroid drug residues in food animals. Bioanalysis **4:**2067-2083.

[2] **Peterson, M. E. and C. R. Ward.** 2007. Etiopathologic findings of hyperthyroidism in cats. Veterinary Clinics of North America-Small Animal Practice **37:**633-645.

[3] **Tomer, Y., J. T. Blackard, and N. Akeno.** 2007. Interferon alpha treatment and thyroid dysfunction. Endocrinology and Metabolism Clinics of North America **36:**1051-1066.

[4] **Dye, J. A., M. Venier, L. Zhu, C. R. Ward, R. A. Hites, and L. S. Birnbaum.** 2007. Elevated PBDE levels in pet cats: Sentinels for humans? Environmental Science & Technology **41:**6350-6356.

[5] **Pearce, E. N. and L. E. Braverman.** 2009. Environmental pollutants and the thyroid. Best Practice & Research Clinical Endocrinology & Metabolism **23:**801-813.

[6] **Vanden Bussche, J., H. Noppe, K. Verheyden, K. Wille, G. Pinel, B. Le Bizec, and H. F. De Brabander.** 2009. Analysis of thyreostats: A history of 35 years. Analytica Chimica Acta **637:**2-12.

[7] **Mackenzie CG, Mackenzie JB.** 1943. Effect of sulfonamides and thioureas on the thyroid gland and basal metabolism. Endocrinology **32**:185-193.

[8] **Greer, M. A. 1980.** Antithyroid drugs in the treatment of thyrotoxicosis. Thyroid today **3**: 1-5.

[9] **Yekeler, H.** 2000. Ab initio study on tautomerism of 2-thiouracil in the gas phase and in solution. Journal of Computer-Aided Molecular Design **14:**243-250.

[10] **Surendra Babu, N.** 2013. Theoretical study of stability, tautomerism, equilibrium constants (pKT) of 2-Thiouracil in gas phase and different solvents (water and acetonitrile) by the density functional theory method. American Chemical Science Journal **3**:137-150.

[11] **Courtheyn, D., B. Le Bizec, G. Brambilla, H. F. De Brabander, E. Cobbaert, A. V. de Wiele, J. Vercammen, and K. De Wasch.** 2002. Recent developments in the use and abuse of growth promoters. Analytica Chimica Acta **473**:71-82.

[12] **De Brabander, H. F.** 1984. Bepalingsmethoden voor thyreostatica in biologisch materiaal. Thesis Ghent University, Ghent, Belgium.

[13] **Terplan, G, L. Kotter, B. Rolle, and H. Geist.** 1964. Zur verwendung des thyreostatikums methylthiouracil in der rindermast. Fleischwirtschaft **44:**457-461.

[14] **Vos, J. G., R. W. Stephany, J. W. Caspers, J. T. G. Van Loon, J. W. H. Metzlar, and H. B. M. Overhaus.** 1982. Weight increase of the thyroid gland as a tentative screening parameter to detect the illegal use of thyreostatic compounds in slaughter cattle. The Veterinary quarterly **4**:1-4.

[15] **Gissel, H. C., M. Schaal.** 1974. Eine dünneschichtchromatografische untersuchungsmethode zum nachweis von thiouracilderivaten in der schilddrüse schlachtbarer haustiere. Archiv für lebensmittelhygiene **1:** 8-13.

[16] **Martinez-Frias, M. L., A. Cereijo, E. Rodriguez-Pinilla, M. Urioste.**1992. Methimazole in animal feed and congenital aplasia cutis. The Lancet **339:**742-743.

[17] **Eghbalian, F.** 2007. Aplasia custis congenita after methimazole exposure in utero; A case report and literature review. The Iranian Journal of Pediatrics **17**:293-296.

[18] **International Agency for Research on Cancer.** 2010. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Available online on: http://monographs.iarc.fr/ENG/Classification/index.php.

[19] **Koninklijk Besluit 12.04.1974**. 1974. Betreffende sommige verrichtingen in verband met stoffen met hormonale, anti-hormonale, anabole, beta-adrenergische, anti-infectieuze, anti-parasitaire en anti-inflammatoire werking. Last consulted in november, 2014. Available online on: http://www.ejustice.just.fgov.be/cgi_loi/change_lg.pl?language=nl&la=N&cn=1974041230&table_name=wet.

[20] **European Community.** 1981. Council Directive 81/602/EC. Official Journal of the European Community **L 222**:32-33.

[21] **Tripathi, M. K. and A. S. Mishra.** 2007. Glucosinolates in animal nutrition: A review. Animal Feed Science and Technology **132:**1-27.

[22] **Bones, A. M. and J. T. Rossiter.** 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. Physiologia Plantarum **97:**194-208.

[23] **Zhou, C. H., J. G. Tokuhisa, D. R. Bevan and A. Esen.** 2012. Properties of beta-thioglucoside hydrolases (TGG1 and TGG2) from leaves of *Arabidopsis thaliana*. Plant Science **191:**82-92.

[24] **Astwood, E. B., M. A. Greer, and M. G. Ettlinger.** 1949. 1-5-Vinyl-2-thiooxazolidone, an antithyroid compound from yellow turnip and from Brassica seeds. The Journal of biological chemistry **181:** 121-130.

[25] **Van Etten, C. H. and I. A. Wolff.** 1973. Natural sulfur compounds. In: Toxicants Occurring Naturally in Foods 2nd edition, Committee on Food Protection, National Academy of Sciences, Washington, DC, USA, 210-234.

[26] **Fenwick, G. R., R. K. Heaney, and W. J. Mullin.** 1983. Glucosinolates and their breakdown products in food and food plants. Critical reviews in food science and nutrition **18:**123-201.

[27] **Fahey, J. W., A. T. Zalcmann, and P. Talalay.** 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56:**5-51.

[28] **Rosa, E. A. S. and A. S. Rodrigues.** 2001. Total and individual glucosinolate content in 11 broccoli cultivars grown in early and late seasons. Hortscience **36:**56-59.

[29] **Halkier, B. A. and L. Du.** 1997. The biosynthesis of glucosinolates. Trends in plant Science **2:**425-431.

[30] **Mithen, R.** 2001. Glucosinolates-biochemistry, genetics and biological activity. Plant Growth Regulation **34:**91-103.

[31] **Mikkelsen, M. D., B. L. Petersen, C. E. Olsen, and B. A. Halkier**. 2002. Biosynthesis and metabolic engineering of glucosinolates. Amino Acids **22:**279-295.

[32] **Wiesner, M., M. Schreiner, and R. Zrenner.** 2014. Functional identification of genes responsible for the biosynthesis of 1-methoxy-indol-3-ylmethyl-glucosinolate in *Brassica rapa ssp. chinensis*. BMC Plant Biology **14:**124.

[33] **Kjaer, A.** 1960. Naturally derived isothiocyanate (mustard oil) and their parent glucosides. In: Progress in the Chemistry of Organic Natural Products, Hetz W, Grisebach H, Kirby GW, Springer-Verlag, Berlin, 122.

[34] **Chen, S. and E. Andreasson.** 2001. Update on glucosinolate metabolism and transport. Plant physiology and biochemistry **39:**743-758.

[35] **Clarke, D. B.** 2010. Glucosinolates, structures and analysis in food. *Analytical Methods* **2**:310-325.

[36] **Lin, L. Z., J. H. Sun, P. Chen, R. W. Zhang, X. E. Fan, L. W. Li, and J. M. Harnly.** 2014. Profiling of glucosinolates and flavonoids in *Rorippa indica (Linn.) Hiern.* (Cruciferae) by UHPLC-PDA-ESI/HRMS^n. Journal of Agricultural and Food Chemistry **62:**6118-6129.

[37] **Zukalova, H. and J. Vasak.** 2002. The role and effects of glucosinolates of Brassica species - a review. Rostlinna Vyroba **48:**175-180.

[38] **Greer, M. A. and J. M. Deeney.** 1959. Antithyroid activity elicited by the ingestion of pure progoitrin, a naturally occurring thioglycoside of the turnip family. The Journal of clinical investigation **38:**1465-1474.

[39] **Krul, C., C. Humblot, C. Philippe, M. Vermeulen, M. Van Nuenen, R. Havenaar, and S. Rabot.** 2002. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic in vitro large-intestinal model. Carcinogenesis **23:**1009-1016.

[40] **Rabot, S., L. Nugon-Baudon, P. Raibud, and O. Szylit.** 1993. Rapeseed meal toxicity in gnotobiotic rats; influence of a whole human fecal flora or single human strains of *Escherichia coli* and *Bacteroides vulgates*. The British journal of nutrition **70:**323-331.

[41] **Palop, M. L. , J. P. Smiths, and B. Ten Brink.** 1995 Degradation of sinigrin by *Lactobacillus agilis* strain R16. International Journal of Food Microbiology **26:**219-229.

[42] **Elfoul, L., S. Rabot, N. Khelifa, A. Quinsac, A. Duguay, and A. Rimbault.** 2001. Formation of allyl isothiocyanate from sinigrin in the digestive tract of rats monoassociated with a human colonic strain of *Bacteroides thetaiotaomicron*. FEMS microbiology letters **197:**99-103.

[43] **Cheng, D. L., K. Hashimoto, and Y. Uda.** 2004. In vitro digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. Food and Chemical Toxicology **42:**351–357.

[44] **Astwood, E**. 1975. In: Pharmacological Basis of Therapeutics, L. S. Goodman, A. Gilman, MacMillan Publishing Co. Inc., New York, USA, 1411.

[45] **Maheshwari PN, Stanley DW, Gray JI, Van de Voort FR.** 1979. An HPLC method for simultaneous quantitation of individual isothiocyanates and oxazolidinethione in myrosinase digests of rapeseed meal. Journal of the American Oil Chemists' Society **56:**837-841.

[46] **De Brabander, H. F. and R. Verbeke.** 1982. Determination of oxazolidine-2-thiones in biological fluids in the ppb range. Journal of Chromatography A **252:**225-239.

[47] **Vermorel, M., M. J. Davicco, and J. Evrard.** 1987. Valorization of rapeseed meal 3. Effects of glucosinolate content on food intake, weight gain, liver weight and plasma thyroid hormone levels in growing rats. Reproduction, nutrition, development **27:**57-66.

[48] **Quinsac, A. and D. Ribaillier.** 1992. Analysis of 5-vinyl-1,3-oxazolidine-2-thione by liquid chromatography. Journal of AOAC International **75:**529-536.

[49] **Mabon, N., J.-P. Wathelet, and M. Marlier.** 1999. Analysis of 5-vinyl-1,3-oxazolidine-2-thione in complex matrices at ppb level. Talanta **49:**199-206.

[50] **Burke, G.** 1970. Effects of cations and ouabain on thyroid adenyl cyclase. Biochimica et Biophysica Acta **220:**30-41.

[51] **Burrow, G. N., W. R. Burke, J. M. Himmelhoch, and R. P. Spencer**. 1971. Effect of lithium on thyroid function. Journal of Clinical Endocrinology & Metabolism **32:**647-652.

[52] **Williams, J. A., S. C. Berens, and J. Wolff.** 1971. Thyroid secretion *in vitro*: Inhibition of TSH and dibutyryl cyclic-AMP stimulated ^{131}I release by Li^+. Endocrinology **88:**1385-1388.

[53] **Männistö, P. T., J. Leppäluoto, and P. Virkkunen.** 1973. Complex antithyroid properties of the lithium ion. Acta Endocrinologica **74:**492-500.

[54] **Lazarus, J. H., A. R. Richards, G. M. Addison, and G. H. Owen.** 1974. Treatment of thyrotoxicosis with lithium carbonate. The Lancet **16:**1160-1163.

[55] **Dhawan, D., R. R. Sharma, and R. J. Dash.** 1988. Effect of short-term and long-term lithium treatment on uptake and retention of iodine-131 in rat thyroid. Australian journal of biological sciences **41:**387-392.

[56] **Greer, M. A., A. K. Stott, and K. A. Milne.** 1966. Effect of thiocyanate, perchlorate and other anions on thyroidall iodine metabolism. Endocrinology **79:**237-247.

[57] **Kubrakova, S.** 1984. Use of perchloric acid salts in animal husbandry. Sbornik Nauchnykh Trudov Moskovskoi Veterinarnoi Adademii 60-64.

[58] **Izhboldina, S. N. and N. E. Malinina.** 1984. Use of perchloric acid salts in animal husbandry. Sbornik Nauchnykh Trudov Moskovskoi Veterinarnoi Adademii 57-60.

[59] **Santuryan, F. Z.** 1986. Effect of ammonium perchlorate in the diet on the function of thyroid gland in mink. Moskovskaya Moskovaskaya Veterinarnaya Adadamiya 89-91.

[60] **Yamada, T. and A. E. Lewis.** 1968. Studies on the mechanism of the inhibitory action of excess iodide on the release of radioiodine from the rat thyroid gland. Endocrinology **82:**54-61.
[61] **Gilman, A. G. and F. Marad.** 1975. Thyroid and antithyroid drugs. The Pharmaceutical Basis of Therapeutics **67:**1398-1420.
[62] **Roepke, R. R., T. H. Maren, and E. Mayer.** 1957. Experimental investigations of sulfamethoxypiridazine. Annals of the New York Academy of Sciences **69:**457-472.
[63] **Milne, K. A. and Greer M. A.** 1962. Clarification of the diverse actions of iodide on antithyroid effect of sulfonamides and thionamides. Proceedings of the Society for Experimental Biology and Medicine **109:**174-176.
[64] **Lagler, F., R. Kretzscmar, F. Leuschner, E. Foitzik, H. Kiel, J. Kuhne, and W. Neumann.** 1976. Toxikologische Untersuchungen der Kombination Sulfamnoxol/Trimethoprim (CN 3123), eines neuen Breitbandchemotherapeutikums. Arzneimittel-Forschung **26:**638-643.
[65] **Trepanier, L. A.** 1999. Delayed hypersensitivity reactions to sulphonamides: syndromes, pathogenesis and management. Veterinary Dermatology **10:**241-248.
[66] **Astwood, E. B.** 1943. The Chemical nature of compounds, which inhibit the function of the thyroid gland. The Journal of pharmacology and experimental therapeutics **78:**79-89.
[67] **Heany, R. K. and G. R. Fenwick.** 1995. Natural toxins and protective factors in brassica species, including rapeseed. Natural Toxins **3:**233-237.
[68] **Bürgi, H.** 2010. Iodine efficiency. Best Practice & Research Clinical Endocrinology & Metabolism **24:**107-115.
[69] **Pinel, G., S. Mathieu, N. Cesbron, D. Maume, H. F. De Brabander, F. Andre, and B. Le Bizec.** 2006. Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyreostats in meat production. Food Additives and Contaminants **23:**974-980.
[70] **European Community.** 1985. Council Directive 85/358/EC. Official Journal of the European Community **L 191:**46-49.
[71] **European Community.** 1996. Council Directive 96/22/EC. Official Journal of the European Community **L 125:**3-9.
[72] **European Community.** 1996. Council Directive 96/23/EC. Official Journal of the European Community **L 125:**10-32.
[73] **European Union.** 2010. Commission Regulation (EU) No. 37/2010. Official Journal of the European Union **L 15:**1-72.
[74] **European Community.** 1990. Council Directive 2377/90. Official Journal of the European Community **L 224:**1-8.
[75] **European Union Reference Laboratories.** 2007. EURL Guidance Document. Available online on: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf.
[76] **European Union.** 2009. Commission Regulation No 470/2009. Official Journal of the European Union **L 152:**11-22.
[77] **European Food Safety Authority (EFSA)-Panel on Contaminants in the Food Chain (CONTAM).** 2013. Guidance of the Scientific Committee/Scientific Panel. European Food Safety Authority Journal **11:**3195 [24 pp.] doi:10.2903/j.efsa.2013.3195.
[78] **Le Bizec, B., E. Bichon, Y. Deceuninck, S. Prevost, F. Monteau, J. P. Antignac, and G. Dervilly-Pinel.** 2011. Toward a criterion for suspect thiouracil administration in animal husbandry. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment **28:**840-847.
[79] **European Community.** 2002. Commissions Decision 2002/657/EC. Official Journal of the European Community **L 221:**8-36.
[80] **Pinel, G., E. Bichon, K. Pouponneau, D. Maume, F. Andre, and B. Le Bizec.** 2005. Multi-residue method for the determination of thyreostats in urine samples using liquid chromatography coupled to tandem mass spectrometry after derivatisation with 3-iodobenzylbromide. Journal of Chromatography A **1085**:247-252.
[81] **Abuin, S., F. Centrich, A. Rubies, R. Companyo, and M. D. Prat.** 2008. Analysis of thyreostatic drugs in thyroid samples by Ultra-Performance Liquid Chromatography tandem mass spectrometry detection. Analytica Chimica Acta **617:**184-191.
[82] **Lõhmus, M., K. Kallaste, and B. Le Bizec.** 2009. Determination of thyreostats in urine and thyroid gland by ultra high performance liquid chromatography tandem mass spectrometry. Journal of Chromatography A **1216:**8080-8089.
[83] **Vanden Bussche, J., L. Vanhaecke, Y. Deceuninck, K. Verheyden, K. Wille, K. Bekaert, B. Le Bizec, and H. F. De Brabander.** 2010. Development and validation of an ultra-high performance liquid chromatography tandem mass spectrometry method for quantifying thyreostats in urine without derivatisation. Journal of Chromatography A **1217:**4285-4293.
[84] **Wauters, J., J. Vanden Bussche, B. Le Bizec, J. A. L. Kiebooms, G. Dervilly-Pinel, S. Prevost, B. Wozniak, S. S. Sterk, D. Gronningen, D. Kennedy, S. Russell, P. Delahaut, L. Vanhaecke.** 2014. Towards a new European threshold to discriminate illegally administered from naturally occurring thiouracil in livestock. Journal of Agricultural and Food Chemistry, submitted.
[85] **Power, D. M., L. Llewellyn, M. Faustino, M. A. Nowell, B. T. Bjornsson, I. E. Einarsdottir, A. V. M. Canario, and G. E. Sweeney.** 2001. Thyroid hormones in growth and development of fish. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **130:**447-459.
[86] **Sjaastad, Ø. V., K. Hove, and O. Sand.** 2004. The endocrine system. In: Physiology of the domestic animals, 2nd ed., Scandinavian Veterinary Press, Oslo, pp. 200-232. ISBN: 82-91743-11-8.

[87] **Singh, R. and S. A. Beigh.** 2013. Diseases of thyroid in animals and their management, insights from veterinary medicine, Dr. Rita Payan Carreira Ed., InTech, ISBN: 978-953-51-1005-7, doi: 10.5772/55377. Available from: http://www.intechopen.com/books/insights-from-veterinary-medicine/diseases-of-thyroid-in-animals-and-their-management.
[88] **Yen, P. M.** 2001. Physiological and molecular basis of thyroid hormone action. Physiological Reviews **81:**1097-1142.
[89] **Kahn, C. M., S. Line & others.** 2005. Endocrine system, The thyroid gland, In: Merck Veterinary Manual. Merck & Co., 9th ed., Inc. Whitehouse station, New Jersey, pp. 460-467.
[90] **Cooper, D. S.** 1984. Antithyroid drugs. New England Journal of Medicine **311:**1353-1362.
[91] **Capen, C. C.** 1992. Pathophysiology of chemical injury of the thyroid gland. Toxicology Letters **64:**381-388.
[92] **Cooper, D. S.** 2005. Drug therapy: Antithyroid drugs. New England Journal of Medicine **352:**905-917.
[93] **Brucker-Davis, F.** 1998. Effects of environmental synthetic chemicals on thyroid function. Thyroid **8:**827-856.
[94] **Traka, M. and R. Mithen.** 2009. Glucosinolates, isothiocyanates and human health. Phytochemistry Reviews **8:**269-282.
[95] **Simopoulos, A. P.** 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine & Pharmacotherapy **56:**365-379.
[96] **Wittkop, B., R. J. Snowdon, and W. Friedt.** 2009. Status and perspectives of breeding for enhanced yield and quality of oilseed crops for Europe. Euphytica **170:**131-140.
[97] **Ministerie van de Vlaamse Gemeenschap - Beleidsdomein Landbouw en Visserij.** 2005. Koolzaad, van zaad tot olie. Available online on: http://www2.vlaanderen.be/landbouw/downloads/plant/koolzaad_van_zaad_tot_olie.pdf.
[98] **Downey, R. K.** 2004. Rapeseed to Canola: Rags to Riches. Agriculture and Agri-Food Canada Research Centre, Saskatoon, SK: 67-76.
Available online on:http://nabc.cals.cornell.edu/Publications/Reports/nabc_18/18_complete.pdf.
[99] **Tripathi, M. K., I. S. Agrawal, S. D. Sharma, and D. P. Mishra.** 2001. Effect of untreated, HCl treated or copper and iodine supplemented high glucosinolate mustard (*Brassica juncea*) meal on nutrient utilization, liver enzymes, thyroid hormones and growth of calves. Animal Feed Science and Technology **92:**73-85.
[100] **Mabon, N., S. N. M. Mandiki, G. Derycke, J.-L. Bister, J. P. Wathelet, R. Paquay, and M. Marlier.** 2000. Chemical changes and influence of rapeseed antinutritional factor on lamb physiology and performance, Part 3. Antinutritional factor in plasma and organ. Animal Feed Science and Technology **85:**111-120.
[101] **Mandiki, S. N. M., N. Mabon, G. Derycke, J.-L. Biste, J. P. Wathelet, R. Paquy, and M. Marlier.** 1999. Chemical changes and influences of rapeseed antinutritional factors on lamb physiology and performance, Part 2. Plasmatic substances and activity of the thyroid. Animal feed science and technology **81:**93-103.
[102] **Downey, R.K. and B. M. Craig.** 1963. Genetic control of fatty acid biosynthesis in rapeseed (*Brassica napus* L.). Journal of the American Oil Chemists' Society **41:**475-478.
[103] **Röbbelen, G.** 1976. Breeding and production of quality rapeseed in Europe. Fette, Seifen, Anstrichmittel, **78:**10-17.
[104] **Aytaç, S., S. Gizlenci, M. Acar, and A. Üstün.** 2006. Changes in erucic acid concentration of rape seeds in advanced generations. Online Journal of Biological Sciences **6:**71-73.
[105] **Bourdon, D. and A. Aumaître.** 1990. Low-glucosinolate rapeseeds and rapeseed meals: effect of technological treatments on chemical composition, digestible energy content and feeding value for growing pigs Animal Feed Science and Technology **30:**175-191.
[106] **European Community.** 2003. Commission Regulation (EC) No 1035/2003. Official Journal of the European Community **L 150:**24-25.
[107] **European Community.** 2002. Council Directive 2002/32/EC. Official Journal of the European Community **L 140:**10-22.
[108] **Cartea, M. E., P. Velasco, S. Obregon, G. Padilla, and A. de Haro.** 2008. Seasonal variation in glucosinolate content in Brassica oleracea crops grown in northwestern Spain. Phytochemistry **69:**403-410.
[109] **Mandiki, S. N. M., G. Derycke, J. L. Bister, N. Mabon, J. P. Wathelet, M. Marlier, and R. Paquay.** 2002. Chemical changes and influences of rapeseed antinutritional factors on gestating and lactating ewes, 1. Animal performances and plasma hormones and glucose. Animal Feed Science and Technology **98:**25-35.
[110] **Box, G. E. and J. S. Hunter.** 1961. 2k-P Fractional Factorial Designs Part I. Technometrics **3:**311-351.
[111] **deAguiar, P. F., B. Bourguignon, M. S. Khots, D. L. Massart, and R. Phan-Than-Luu.** 1995. D-optimal designs. Chemometrics and Intelligent Laboratory Systems **30:**199-210.
[112] **Beres, D. L. and D. M. Hawkins.** 2001. Plackett-Burman techniques for sensitivity analysis of many parametered models. Ecological Modelling **141:**171-183.
[113] **Christian, G. D., P. K. Dasgupta, and K. A. Schug.** 2014. Analytical chemistry, 7th ed., Wiley, Hoboken. pp. 649-765. ISBN: 978-0-470-88757-8.
[114] **Swartz, M. E. 2005.** UPLC: An introduction and review. Journal of Liquid Chromatography & Related Technologies **28:**1253-1263.
[115] **Wu, N. and A. M Clausen.** 2007. Fundamental and practical aspects of ultrahigh pressure liquid chromatography for fast separations. Journal of Separation Sciences **30:**1167-1182.

[116] **Novakova, L., L. Matysova, and P. Solich.** 2006. Advantages of application of UPLC in pharmaceutical analysis. Talanta **68:**908-918.
[117] **Waters Corporation, USA.** 2005. Ultra performance LC, separation science redefined. 720001136EN.
[118] **Stolker, A. A. M., T. Zuidema, and M. W. F. Nielen.** 2007. Residue analysis of veterinary drugs and growth-promoting agents. Trends in Analytical Chemistry **26:**967-979.
[119] **De Brabander, H. F., B. Le Bizec, G. Pinel, J.-P. Antignac, K. Verheyden, V. Mortier, D. Courtheyn, and H. Noppe.** 2007. Past, present and future of mass spectrometry in the analysis of residues of banned substances in meat-producing animals. Journal of Mass Spectrometry **42:**983-998.
[120] **Agilent Technologies, USA.** 2000. Liquid Chromatography/ Mass Spectrometry, Development of a new orthogonal geometry atmospheric pressure ionization interface for LC/MS. (23) 5968-4465E.
[121] **Manisali, I., D. D. Y. Chen, and B. B. Schneider.** 2006. Electrospray ionization source geometry for mass spectrometry: past, present, and future. Trac-Trends in Analytical Chemistry **25:**243-256.
[122] **Ni, J. S., O. Y. Hui, M. Aiello, C. Seto, L. Borbridge, T. Sakuma, R. Ellis, D. Welty, and A. Acheampong.** 2008. Micro dosing assessment to evaluate pharmacokinetics and drug metabolism in rats using liquid chromatography-tandem mass spectrometry. Pharmaceutical Research **25:**1572-1582.
[123] **Banerjee, S. and S. Mazumdar**. 2012. Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. International Journal of Analytical Chemistry **2012**:40 pages, doi:10.1155/2012/282574.
[124] **Willoughby, R., E. Sheehan, and S. Mitrovich.** 2002. Is LC-MS right for you? In: A global view of LC-MS. 2nd ed., Global View Publishing, Pittsburgh, pp. 44-89.
[125] **March, R. E.** 1997. An introduction to quadrupole ion tram mass spectrometry. Journal of Mass Spectrometry **32:**351-369.
[126] **Lurye, J. C., E. N. Behrend, and R. J. Kemppainen.** 2002. Evaluation of an in-house enzyme-linked immunosorbent assay for quantitative measurement of serum total thyroxine concentration in dogs and cats. Journal of the American Veterinary Medical Association **221:**243-249.
[127] **Griem, W.** 1973. Histopathological changes in the thyroid glands of cattle and rabbits following feeding with methylthiouracil. Berliner und Munchener Tierarztliche Wochenschrift **86:**50-56.
[128] **Pottie, G.** 1979. Verwendbarkeit der histologischen schilddrüsenuntersuchung bei erwachsenen rindern zur ermittlung des mißbrauches von thyreostatica. Fleischwirtschaft **59:**248-250.
[129] **Van Waes, H**. 1973. Bepaling van methylthiouracil in vlees. Landbouwtijdschrift **2:**437-441.
[130] **De Brabander, H. F. and R. Verbeke.** 1975. Detection of antithyroid residues in meat and some organs of slaughtered animals. Journal of chromatography **108:**141-151.
[131] **De Brabander, H. F., P. Batjoens, and J. Van Hoof.** 1992. Determination of thyreostatic drugs by HPTLC with confirmation by GC-MS. Journal of Planar Chromatography **5:**24-130.
[132] **De Brabander, H. F. , F. Smets, and G. Pottie G.** 1988. Faster and cheaper two-dimensional HPTLC using the "4 x4" mode. Journal of Planar Chromatography **1:**369-371.
[133] **De Brabander H. F., G. Rasschaert, and W. H. Vanneste.** 1989. An Automated "4 x4" Sample Applicator. Journal of Planar Chromatography **2:**484-485.
[134] **Floberg, S., K. Lanbeck, and B. Lindstrom.** 1980. Determination of methimazole in plasma using gas chromatography-mass spectrometry after extractive alkylation. Journal of Chromatography **182:**63-70.
[135] **Buhlert, J.** 1986. A method to determine thyrostatics through gas-chromatography. Deutsche Lebensmittel-Rundschau **820:**146-148.
[136] **Schilt, R., J. M. Weseman, H. Hooijerink, H. J. Korbee, W. A. Traag, M. J. Van Steenbergen, and W. Haasnoot.** 1989. Screening and confirmation of thyreostatics in urine by gas chromatography with nitrogen-phosphorus detection and gas chromatography-mass spectrometry after sample clean-up with a mercurated affinity column. Journal of Chromatography **489:**127-137.
[137] **Pochard, M. F.** 1988. Plan de contrôle anabolisants-thyréostatiques DGAL/SVHA/N88/N°8057.
[138] **Le Marrec, N.** 1995. Analyse des antithyroïdiens de synthèse en GC/MS et LC/MS: application au contrôle de leur présence chez les animaux de boucherie, Thèse d'Université, Nantes, France.
[139] **Yu, G. Y. F., E. J. Murby, and R. J. Wells.** 1997. Gas chromatographic determination of residues of thyreostatic drugs in bovine muscle tissue using combined resin mediated methylation and extraction. Journal of Chromatography B **703:**159-166.
[140] **Batjoens, P., H. F. De Brabander, and K. De Wasch.** 1996. Rapid and high-performance analysis of thyreostatic drugs residues in urine using gas chromtography-mass spectrotmetry. Journal of Chromatography A **750:**127-132.
[141] **Le Bizec, B., F. Monteau, D. Maume, M. Montrade, C. Gade, and F. Andre.** 1997. Detection and identification of thyreostats in the thyroid gland by gas chromatograpy-mass spectrometry. Analytica Chimica Acta **340:**201-208.
[142] **Zou, Q., Y. Liu, M. Xie, J. Han, and L. Zang.** 2005. A rapid method for determination and confirmation of the thyreostats in milk and urine by matrix solid-phase dispersion and gas chromatography-mass spectrometry. Analytica Chimica Acta **551:**184-191.
[143] **Zhang, L., Y. Liu, M. Xie, and Y. Qiu.** 2005. Simultaneous determination of thyreostatic residues in animal tissues by matrix solid-phase dispersion and gas Chromatography-mass spectrometry. Journal of Chromatography A **1074:**1-7.
[144] **Le Bizec, B., J. P. Antignac, E. Bichon, G. Pinel, and K. Worrall.** 2005. Application of GC-MS/MS for the analysis of banned growth promoters. WATERS Applications Note 720001124EN.

[145] **Wildanger, W.** 1975. Separation of some thyreostatics by high-pressure liquid chromatography. Chromatographia **8:**42-44.
[146] **Henning, W.** 1986. Lebensmittelchemie Gerichtlung Chemie **40:**1-4.
[147] **Skellern, G. G., B. I. Kinght, and J. B. Stenlake.** 1976. Improved method for the determination of methimazole in plasma by high performance liquid chromatography. Journal of Chromatography **124:**405-410.
[148] **Hooijerink, H. and W. G. De Ruig.** 1987. Determination of thyreostatics in meat by reversed-phase liquid chromatography with ultraviolet and electrochemical detection. Journal of Chromatography **394:**403-407.
[149] **Moretti, G., P. Betto, P. Cammarata, F. Fracassi, M. Giambenedetti, and A. Borghese.** 1993. Determination of thyreostatic residues in cattle plasma by high-performance liquid-chromatography with ultraviolet detection. Journal of Chromatography-Biomedical Applications 616: 291-296.
[150] **Wei, Y., Z.-J. Zhang, Y.-T. Zhang, and Y.-H. Sun.** 2007. Determination of propylthiouracil and methylthiouracil in human serum using high-performance liquid chromatography with chemiluminescence detection. Journal of Chromatography B **854:**239-244.
[151] **Buick, R. K., C. Barry, I. M. Traynor, W. J. McCaughey, and C. T. Elliott.** 1998. Determination of thyreostat residues from bovine matrices using high-performance liquid chromatography. Journal of chromatography B **720:**71-79.
[152] **Blanchflower, W. J., P. J. Hughes, A. Cannavan, M. A. McCoy, and D. G. Kennedy.** 1997. Determination of thyreostats in thyroid and urine using High-performance Liquid Chromatography-Atmospheric Pressure Chemical Ionisation Mass Spectrometry. The Analyst **122:**967-972.
[153] **De Wasch, K., H. F. De Brabander, S. Impens, M. Van de Wiele, and D. Courtheyn.** 2001. Determination of mercaptobenzimidazol and other thyreosta residues in thyroid tissue and meat using high-performance liquid chromatography-mass spectrometry. Journal of Chromatography A **912:**311-317.
[154] **De Wasch, K., H. F. De Brabander, L. A. Van Ginkel, A. Spaan, S. S. Sterk, and H. D. Meiring.** 1998. Confirmation of residues of thyreostatic drugs in thyroid glands by multiple mass spectrometry after thin-layer chromatographic screening. Journal of Chromatography A **819:**99-111.
[155] **Prasad, B., A. Garg, H. Takwani, and S. Singh.** 2011. Metabolite identification by liquid chromatography-mass spectrometry. Trac-Trends in Analytical Chemistry **30:**360-387.
[156] **Hu, Q. Z., R. J. Noll, H. Y. Li, A. Makarov, M. Hardman, and R. G. Cooks.** 2005. The Orbitrap: a new mass spectrometer. Journal of Mass Spectrometry **40:**430-443.
[157] **Fedorova, G., T. Randak, R. H. Lindberg, and R. Grabic.** 2013. Comparison of the quantitative performance of a Q-Exactive high-resolution mass spectrometer with that of a triple quadrupole tandem mass spectrometer for the analysis of illicit drugs in wastewater. Rapid Communications in Mass Spectrometry **27:**1751-1762.
[158] **Herrero, P., N. Cortes-Francisco, F. Borrull, J. Caixach, E. Pocurull, and R. M. Marce.** 2014. Comparison of triple quadrupole mass spectrometry and Orbitrap high-resolution mass spectrometry in ultrahigh performance liquid chromatography for the determination of veterinary drugs in sewage: benefits and drawbacks. Journal of Mass Spectrometry **49:**585-596.
[159] **Léon, N., M. Roca, C. Igualada, C. P. B. Martins, A. Pastor, and V. Yusa.** 2012. Wide-range screening of banned veterinary drugs in urine by ultra high liquid chromatography coupled to high-resolution mass spectrometry. Journal of Chromatography A **1258:**55-65.
[160] **Esteve-Romero, J., I. Escrig-Tena, E. F. Simo-Alfonso, and G. Ramis-Ramos.** 1999. Determination of thyreostatics in animal feed by micellar electrokinetic chromatography. Analyst **124:**125-128.
[161] **Perez-Ruiz, T., C. Martinez-Lozano, A. Sanz, and R. Galera.** 2005. An ultrasensitive method for the determination of thiouracil and phenylthiouracil using capillary zone electrophoresis and laser-induced fluorescence detection. Electrophoresis **26:**2384-2390.
[162] **Kong, D. X., Y. W. Chi, L. C. Chen, Y. Q. Dong, L. Zhang, and G. N. Chen.** 2009. Determination of thyreostatics in animal feeds by CE with electrochemical detector. Electrophoresis **30:**3489-3495.
[163] **Ebrahimzadeh, H., A. A. Asgharinezhad, L. Adlnasab, and N. Shekari.** 2012. Optimization of ion-pair based hollow fiber liquid phase microextraction combined with HPLC-UV for the determination of methimazole in biological samples and animal feed. Journal of Separation Science **35:**2040-2047.
[164] **Vanden Bussche, J., J. A. L. Kiebooms, N. De Clercq, Y. Deceuninck, B. Le Bizec, H. F. De Brabander, and L. Vanhaecke.** 2011. Feed or Food Responsible for the Presence of Low-Level Thiouracil in Urine of Livestock and Humans? Journal of Agricultural and Food Chemistry **59:**5786-5792.
[165] **Wozniak, B., S. Witek, I. Matraszek-Zuchowska, and J. Zmudzki.** 2014. Determination of the thyreostats in animal feeding stuffs using liquid chromatography-tandem mass spectrometry. Bulletin of the Veterinary Institute in Pulawy **58:**413-419.
[166] **Schachter, S., R. W. Nelson, C. Scott-Moncrieff, D. C. Ferguson, T. Montgomery, E. C. Feldman, L. Neal, and P. H. Kass.** 2004. Comparison of serum-free thyroxine concentrations determined by standard equilibrium dialysis, modified equilibrium dialysis, and 5 radioimmunoassays in dogs. Journal of Veterinary Internal Medicine **18:**259-264.
[167] **Wakeling, J., K. Moore, J. Elliott, and H. Syme.** 2008. Diagnosis of hyperthyroidism in cats with mild chronic kidney disease. Journal of Small Animal Practice **49:**287-294.

[168] **Fazio, E., P. Medica, C. Cravana, and A. Ferlazzo.** 2012. Total and free iodothyronines profile in the donkey (Equus asinus) over a 12-month period. Acta Veterinaria Brno **81:**239-244.
[169] **Soldin, O. P. and S. J. Soldin.** 2011. Thyroid hormone testing by tandem mass spectrometry. Clinical Biochemistry **44:**89-94.
[170] **Thienpont, L. M., K. Van Uytfanghe, K. Poppe, and B. Velkeniers.** 2013. Determinatioon of free thyroid hormones. Best Practice & Researh Clinical Endocrinology & Metabolism **27:**689-700.
[171] **Wu, A. H. B. and D. French.** 2013. Implementation of liquid chromatography/mass spectrometry into the clinical laboratory. Clinica Chimica Acta **420:**4-10.
[172] **Singh, A. K., Y. Jiang, T. White, and D. Spassova.** 1997. Validation of nonradioactive chemiluminescent immunoassay methods for the analysis of thyroxine and cortisol in blood samples obtained from dogs, cats, and horses. Journal of Veterinary Diagnostic Investigation **9:**261-268.
[173] **Kemppainen, R. J. and J. R. Birchfield.** 2006. Measurement of total thyroxine concentration in serum from dogs and cats by use of various methods. American Journal of Veterinary Research **67:**259-265.
[174] **Sapin, R. and M. d'Herbomez.** 2003. Free thyroxine measured by equilibrium dialysis and nine immunoassays in sera with various serum thyroxine-binding capacities. Clinical Chemistry **49:**1531-1535.
[175] **Steele, B. W., E. Wang, G. G. Klee, L. M. Thienpont, S. J. Soldin, L. J. Sokoll, W. E. Winter, S. A. Fuhrman, and R. J. Elin.** 2005. Analytic bias of thyroid function tests - Analysis of a College of American Pathologists fresh frozen serum pool by 3900 clinical laboratories. Archives of Pathology & Laboratory Medicine **129:**310-317.
[176] **De Brabandere, V. I., P. Hou, D. Stockl, L. M. Thienpont, and A. P. De Leenheer.** 1998. Isotope dilution-liquid chromatography electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method. Rapid Communications in Mass Spectrometry **12:**1099-1103.
[177] **Holm, S. S., S. H. Hansen, J. Faber, and P. Staun-Olsen.** 2004. Reference methods for the measurement of free thyroid hormones in blood evaluation of potential reference methods for free thyroxine. Clinical Biochemistry **37:**85-93.
[178] **Van Uytfanghe, K., D. Stockl, H. A. Ross, and L. M. Thienpont.** 2006. Use of frozen sera for FT4 standardization: Investigation by equilibrium dialysis combined with isotope dilution-mass spectrometry and immunoassay. Clinical Chemistry **52:**1817-1820.
[179] **Yue, B. F., A. L. Rockwood, T. Sandrock, S. L. La'ulu, M. M. Kushnir, and A. W. Meikle.** 2008. Free thyroid hormones in serum by direct equilibrium dialysis and online solid-phase extraction-liquid chromatography/tandem mass spectrometry. Clinical Chemistry **54:**642-651.
[180] **Hoofnagle, A. N. and M. H. Wener.** 2009. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. Journal of Immunological Methods **347:**3-11.
[181] **Wang, D. L. and H. M. Stapleton.** 2010. Analysis of thyroid hormones in serum by liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry **397:**1831-1839.
[182] **De Brabandere, V. I., D. Stockl, L. M. Thienpont, and A. P. De Leenheer.** 1998. On the use of trimethylchlorosilane in methanol for methylation of thyroxine prior to perfluoroacylation and isotope dilution gas chromatography mass spectrometry. Journal of Mass Spectrometry **33:**1032-1032.
[183] **Thienpont, L. M., C. Fierens, A. P. De Leenheer, and L. Przywara.** 1999. Isotope dilution-gas chromatography/mass spectrometry and liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of triiodo-L-thyronine in serum. Rapid Communications in Mass Spectrometry **13:**1924-1931.
[184] **Soukhova, N., O. P. Soldin, and S. J. Soldin.** 2004. Isotope dilution tandem mass spectrometric method for T4/T3. Clinica Chimica Acta **343:**185-190.
[185] **Tai, S. S. C., L. T. Sniegoski, and M. J. Welch.** 2002. Candidate reference method for total thyroxine in human serum: Use of isotope-dilution liquid chromatography-mass spectrometry with electrospray ionization. Clinical Chemistry **48:**637-642.
[186] **Tai, S. S. C., D. M. Bunk, E. V. White, and M. J. Welch.** 2004. Development and evaluation of a reference measurement procedure for the determination of total 3,3 ',5-trilodothyronine in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Analytical Chemistry **76:**5092-5096.
[187] **Vogl, J. and W. Pritzkow.** 2010. Isotope Dilution Mass Spectrometry - A Primary Method of Measurement and Its Role for RM Certification. Mapan-Journal of Metrology Society of India **25:**135-164.
[188] **Fierens, C., D. Stöckl, L. M. R. Thienpont, and A. P. De Leenheer.** 2000. Towards a reference method for serum free thyroxine based on isotope dilution mass spectrometry. Immuno-analyse & Biologie Spécialisée **15:**311-317.
[189] **Peterson, M. E.** 2006. Diagnostic tests for hyperthyroidism in cats. Clinical Techniques in Small Animal Practice **21:**2-9.
[190] **Gu, J., O. P. Soldin, and S. J. Soldin.** 2007. Simultaneous quantification of free triiodothyronine and free thyroxine by isotope dilution tandem mass spectrometry. Clinical Biochemistry **40:**1386-1391.
[191] **Di, L., J. P. Umland, P. E. Trapa, and T. S. Maurer.** 2012. Impact of recovery of fraction unbound using equilibrium dialysis. Journal of Pharmaceutical Sciences **101:**1327-1335.

[192] **Jonklaas, J., N. Kahric-Janicic, O. P. Soldin, and S. J. Soldin.** 2009. Correlations of Free Thyroid Hormones Measured by Tandem Mass Spectrometry and Immunoassay with Thyroid-Stimulating Hormone across 4 Patient Populations. Clinical Chemistry **55:**1380-1388.

[193] **Soldin, S. J., N. Soukhova, N. Janicic, J. Jonklaas, and O. P. Soldin.** 2005. The measurement of free thyroxine by isotope dilution tandem mass spectrometry. Clinica Chimica Acta **358:**113-118.

[194] **Weeke, J., N. Boye, and H. Orskov.** 1986. Ultrafiltration Method for Direct Radioimmunoassay Measurement of Free-Thyroxine and Free Triiodothyronine in Serum. Scandinavian Journal of Clinical & Laboratory Investigation **46:**381-389.

[195] **Tikanoja, S.** 1990. Ultrafiltration Devices Tested for Use in a Free-Thyroxine Assay Validated by Comparison with Equilibrium Dialysis. Scandinavian Journal of Clinical & Laboratory Investigation **50:**663-669.

[196] **Kararli, T. T.** 1995. Comparison of the Gastrointestinal Anatomy, Physiology, and Biochemistry of Humans and Commonly Used Laboratory-Animals. Biopharmaceutics & Drug Disposition **16:**351-380.

[197] **Frandson, R. D., Spurgeon T. L.** 1992. Digestive system. In: Anatomy and physiology of farm animals. 5th edition, Lippincott Williams and Wilkins, London. ISBN: 0-8121-1435-3. pp. 304-377.

[198] **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Applied and Environmental Microbiology **68:**673-690.

[199] **Bergman, E. N.** 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews **70:**567-590.

[200] **Salanitro, J. P., I. G. Blake, and P. A. Muirhead.** 1977. Isolation and identification of fecal Bacteria from adult swine. Applied and Environmental Microbiology **33:**79-84.

[201] **Russell, E. G.** 1979. Types and distribution of anaerobic bacteria in the large intestine of pigs. Applied and Environmental Microbiology **37:**187-193.

[202] **Robinson, I. M., M. J. Allison, and J. A. Bucklin.** 1981. Characterization of the cecal bacteria of normal pigs. Applied and Environmental Microbiology **41:**950-955.

[203] **Robinson, I. M., S. C. Whipp, J. A. Bucklin, and M. J. Allison.** 1984. Characterization of predominant bacteria from the colons of normal and dysenteric Pigs. Applied and Environmental Microbiology **48:**964-969.

[204] **Mikkelsen, L. L., M. Jakobsen, and B. B. Jensen.** 2003. Effects of dietary oligosaccharides on microbial diversity and fructo-oligosaccharide degrading bacteria in faeces of piglets post-weaning. Animal Feed Science and Technology **109:**133-150.

[205] **Richards, J. D., J. Gong, and C. F. M. de Lange.** 2005. The gastrointestinal microbiota and its role in monogastric nutrition and health with an emphasis on pigs: Current understanding, possible modulations, and new technologies for ecological studies. Canadian Journal of Animal Science **85:**421-435.

[206] **Tajima, K., R. I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui, and Y. Benno.** 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiology Ecology **29:**159-169.

[207] **Sjaastad, Ø. V., K. Hove, O. Sand.** 2004. The digestive system. In: Physiology of the domestic animals. 2nd ed., Scandinavian Veterinary Press, Oslo, pp. 490–563. ISBN: 82-91743-11-8.

[208] **Callaway, T. R., S. E. Dowd, T. S. Edrington, R. C. Anderson, N. Krueger, N. Bauer, P. J. Kononoff, and D. J. Nisbet.** 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. Journal of Animal Science **88:**3977-3983.

[209] **Dowd, S. E., T. R. Callaway, R. D. Wolcott, Y. Sun, T. McKeehan, R. G. Hagevoort, and T. S. Edrington.** 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). Bmc Microbiology **8**.

[210] **Maki, L. R. and K. Picard.** 1965. Normal intestinal flora of cattle fed high roughage rations. Journal of Bacteriology **89:**1244-1249.

[211] **Wickham, M., R. Faulks, and C. Mills.** 2009. In vitro digestion methods for assessing the effect of food structure on allergen breakdown. Molecular Nutrition & Food Research **53:**952-958.

[212] **Gérard-Champod, M., S. Blanquet-Diot, J.-M. Cardot, D. Bravo, and M. Alric.** 2010. Development and validation of a continuous in vitro system reproducing some biotic and antibiotic factors of the veal calf intestine. Applied and Environmental Microbiology **76:**5592-5600.

[213] **Coles, L. T., P. J. Moughan, and A. J. Darragh.** 2005. In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. Animal Feed Science and Technology **123:**421-444.

[214] **Miller, T. L. and M. J. Wolin.** 1981. Fermentation by the human large intestine microbial community in an *in vitro* semicontinuous culture system. Applied and Environmental Microbiology **42:**400-407.

[215] **Veilleux, G. and I. Rowland.** 1981. Simulation of the rat intestinal ecosystem using a two-stage continuous culture system. Journal of General Microbiology **123:**103-115.

[216] **Gibson, G. R., J. H. Cummings, and G. T. Macfarlane.** 1988. Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Applied and Environmental Microbiology **54:**2750-2755.

[217] **Molly, K., M. V. Woestyne, and W. Verstraete.** 1993. Development of a 5-Step Multichamber Reactor as a Simulation of the Human Intestinal Microbial Ecosystem. Applied Microbiology and Biotechnology **39:**254-258.

[218] **Minekus, M., M. Smeets-Peeters, A. Bernalier, S. Marol-Bonnin, R. Havenaar, P. Marteau, M. Alric, G. Fonty, and J. H. J. H. I. Veld.** 1999. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Applied Microbiology and Biotechnology **53:**108-114.
[219] **Oomen, A. G., A. Hack, M. Minekus, E. Zeijdner, C. Cornelis, G. Schoeters, W. Verstraete, T. Van de Wiele, J. Wragg, C. J. M. Rompelberg, A. J. A. M. Sips, and J. H. Van Wijnen.** 2002. Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. Environmental Science & Technology **36:**3326-3334.
[220] **De Boever, P., B. Deplancke, and W. Verstraete.** 2000. Fermentation by gut microbiota cultured in a simulator of the human intestinal microbial ecosystem is improved by supplementing a soygerm powder. Journal of Nutrition **130:**2599-2606.
[221] **Van de Wiele, T., N. Boon, S. Possemiers, H. Jacobs, and W. Verstraete.** 2004. Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. FEMS Microbiology Ecology **51:**143-153.
[222] **Chiang C-C, J. Croom, S.-T. Chusang, P. W. S. Chiou, and B. Yu**. 2008. Development of a dynamic system simulating pig gastric digestion. Asian-Australasion Journal of Animal Science **21:**1552-1528.
[223] **Rivest, J., J. F. Bernier, and C. Pomar.** 2000. A dynamic model of protein digestion in the small intestine of pigs. Journal of Animal Science. **78**:328-340.
[224] **Bastianelli D., D. Sauvant, and A. Rérat**. 1996. Mathematical modeling of digestion and absorption in pigs. Journal of Animal Science. **74**:1873-1887.
[225] **Menke, K. H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider.** 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. The Journal of Agricultural Science **93:**217-222**.**
[226] **Van Nevel, C. J., and D. I. Demeyer.** 1979. Effect of Monensin on Some Rumen Fermentation Parameters. Annales De Recherches Veterinaires **10:**338-340.
[227] **Duncan, A. J. and J. A. Milne.** 1992. Rumen Microbial-Degradation of Allyl Cyanide as a Possible Explanation for the Tolerance of Sheep to Brassica-Derived Glucosinolates. Journal of the Science of Food and Agriculture **58:**15-19.
[228] **Vanmilgen, J., L. L. Berger, and M. R. Murphy.** 1993. An Integrated, Dynamic-Model of Feed Hydration and Digestion, and Subsequent Bacterial Mass Accumulation in the Rumen. British Journal of Nutrition **70:**471-483.
[229] **Van Nevel, C. J. and D. I. Demeyer.** 1996. Influence of pH on lipolysis and biohydrogenation of soybean oil by rumen contents in vitro. Reproduction Nutrition Development **36:**53-63.
[230] **Machmüller, A., D. A. Ossowski, M. Wanner, and M. Kreuzer.** 1998. Potential of various fatty feeds to reduce methane release from rumen fermentation in vitro (Rusitec). Animal Feed Science and Technology **71:**117-130.
[231] **Fievez, V., F. Dohme, M. Danneels, K. Raes, and D. Demeyer.** 2003. Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation in vitro and in vivo. Animal Feed Science and Technology **104:**41-58.
[232] **Boeckaert, C., B. Vlaeminck, J. Mestdagh, and V. Fievez.** 2007. In vitro examination of DHA-edible micro algae 1. Effect on rumen lipolysis and biohydrogenation of linoleic and linolenic acids. Animal Feed Science and Technology **136:**63-79.
[233] **Fievez, V., C. Boeckaert, B. Vlaeminck, J. Mestdagh, and D. Demeyer.** 2007. In vitro examination of DHA-edible micro-algae 2. Effect on rumen methane production and apparent degradability of hay. Animal Feed Science and Technology **136:**80-95.
[234] **Fievez, V., B. Vlaeminck, T. Jenkins, F. Enjalbert, and M. Doreau.** 2007. Assessing rumen biohydrogenation and its manipulation in vivo, in vitro and in situ. European Journal of Lipid Science and Technology **109:**740-756.
[235] **Vlaeminck, B., G. Mengistu, V. Fievez, L. de Jonge, and J. Dijkstra.** 2008. Effect of in vitro docosahexaenoic acid supplementation to marine algae-adapted and unadapted rumen inoculum on the biohydrogenation of unsaturated fatty acids in freeze-dried grass. Journal of Dairy Science **91:**1122-1132.
[236] **Huhtanen, P., A. Seppala, M. Ots, S. Ahvenjarvi, and M. Rinne.** 2008. In vitro gas production profiles to estimate extent and effective first-order rate of neutral detergent fiber digestion in the rumen. Journal of Animal Science **86:**651-659.
[237] **Soliva, C. R., S. L. Amelchanka, S. M. Duval, and M. Kreuzer.** 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). British Journal of Nutrition **106:**114-122.
[238] **Soliva, C. R. and C. Kunz.** 2011. Preliminary study on the effects of ammonium nicotinate on in vitro ruminal fermentation as determined using rumen simulation technique (Rusitec). Animal Production Science **51:**233-239.
[239] **Machmüller, A., C. R. Soliva, and M. Kreuzer.** 2002. In vitro ruminal methane suppression by lauric acid as influenced by dietary calcium. Canadian Journal of Animal Science **82:**233-239.
[240] **Czerkawski, J. W. and G. Breckenridge.** 1977. Design and development of a long-term rumen simulation technique (Rusitec). British Journal of Nutrition **38:**371-384.
[241] **Soliva, C. R. and H. D. Hess.** 2007. Measuring methane emission of ruminants by *in vitro* and *in vivo* techniques. In: Measuring Methane Production from Ruminants, H. P. S. Makkar and P. E. Vercoe, eds. Dordrecht: Springer. pp.13 – 15.

[242] **Dijkstra, J., H. D. S. C. Neal, D. E. Beever, and J. France.** 1992. Simulation of Nutrient Digestion, Absorption and Outflow in the Rumen - Model Description. Journal of Nutrition **122:**2239-2256.
[243] **Maskell, I. and R. Smithard.** 1994. Degradation of Glucosinolates during in-Vitro Incubations of Rapeseed Meal with Myrosinase (Ec-3.2.3.1) and with Pepsin (Ec-3.4.23.1) Hydrochloric acid, and contents of porcine small intestine and cecum. British Journal of Nutrition **72:**455-466.
[244] **Boisen, S. and J. A. Fernandez.** 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by in vitro analyses. Animal Feed Science and Technology **68:**277-286.
[245] **Gawlik-Dziki, U., D. Dziki, B. Baraniak, and R. F. Lin.** 2009. The effect of simulated digestion in vitro on bioactivity of wheat bread with Tartary buckwheat flavones addition. Lwt-Food Science and Technology **42:**137-143.
[246] **Lee, S. S., M. B. Chang, and J. K. Ha.** 2004. Effects of bacterial fraction and proportion of silage and concentrate on rumen fermentation and gas production profile. Asian-Australasian Journal of Animal Sciences **17:**643-647.
[247] **Van Ranst, G., V. Fievez, M. Vandewalle, C. Van Waes, J. De Riek, and E. Van Bockstaele.** 2010. Influence of damaging and wilting red clover on lipid metabolism during ensiling and *in vitro* rumen incubation. Animal **4:**1528-1540.
[248] **Dubos, R. J.** 1946. The adaptive production of enzymes by bacteria. Bacteriology Review **4:**1–16.
[249] **Tortora, G. J., B. R. Funke, and C. L. Case.** 2002. Microbiology an introduction - media update, 7th ed., Benjamin Cummings, London.
[250] **Ohtsuru, M., I. Tsuruo, and T. Hata.** 1973. Studies on Fungous Myrosinase .4. Production and Stability of Intracellular Myrosinase from Aspergillus-Niger. Agricultural and Biological Chemistry **37:**967-971.
[251] **Smits, J. P., W. Knol, and J. Bol.** 1993. Glucosinolate degradation by *Aspergillus clavatus* and *Fusarium oxysporum* in liquid and solid-state fermentation. Applied Microbiology and Biotechnology **38:**696-701.
[252] **Rakariyatham, N. and P. Sakorn.** 2002. Biodegradation of glucosinolates in brown mustard seed meal (*Brassica juncea*) by *Aspergillus sp.* NR-4201 in liquid and solid-state cultures. Biodegradation **13:**395-399.
[253] **Staron, T.** 1975. Obtention des proteines à partir des graines oleagineuses par des methodes microbiologiques. Proteines et Graines Oleagineuses **11-12:**579–589.
[254] **Huber J., G. Kranz, G. Kreibich, K. Beining, M. Krüge, and F. Weissbach.** 1983 Mikrobieller Abbau von Glucosinolaten im Rapsextraktionsschrot. Food/Nahrung **27:**257-263.
[255] **Meulenbeld, G. H. and S. Hartmans.** 2001. Thioglucosidase activity from *Sphingobacterium sp.* strain OTG1. Applied Microbiology and Biotechnology **56:**700-706.
[256] **Tani, N., M. Ohtsuru, and T. Hata.** 1974. Studies on Bacterial Myrosinase .2. Purification and General Characteristics of Bacterial Myrosinase Produced by Enterobacter-Cloacae. Agricultural and Biological Chemistry **38:**1623-1630.
[257] **Rabot, S., C. Guerin, L. Nugon-Baudon, and O. Szylit.** 1995. Glucosinolate degradation by bacterial strains isolated from a human intestinal microflora. Proceedings of the 9th International Rapeseed Congress **1:**212-214.
[258] **Mroczynska, M. and Z. Libudzisz.** 2010. Beta-glucuronidase and beta-glucosidase Activity of Lactobacillus and Enterococcus Isolated from Human Feces. Polish Journal of Microbiology **59:**265-269.
[259] **Li, F., M. A. J. Hullar, Y. Schwarz, and J. W. Lampe.** 2009. Human Gut bacterial communities are altered by addition of cruciferous vegetables to a controlled fruit- and vegetable-free Diet. Journal of Nutrition **139:**1685-1691.
[260] **Oginsky, E. L., A. E. Stein, and M. A. Greer.** 1965. Myrosinase activity in bacteria as demonstrated by conversion of progoitrin to goitrin. Proceedings of the Society for Experimental Biology and Medicine **119:**360-4.
[261] **Nugon-Baudon, L., O. Szylit, and P. Raibaud.** 1988. Production of toxic glucosinolate derivatives from rapeseed meal by intestinal microflora of rat and chicken. Journal of the Science of Food and Agriculture **43:**299-308.
[262] **Nugon-Baudon, L., S. Rabot, J. M. Wal, and O. Szylit.** 1990. Interactions of the Intestinal Microflora with Glucosinolates in Rapeseed Meal Toxicity - 1st Evidence of an intestinal *Lactobacill*us possessing a myrosinase-like activity *in vivo*. Journal of the Science of Food and Agriculture **52:**547-559.
[263] **Li, W. J., D. Raoult, and P. E. Fournier.** 2009. Bacterial strain typing in the genomic era. FEMS Microbiology Reviews **33:**892-916.
[264] **Fakruddin, M. D., K. S. B. Mannan, R. M. Mazumdar, A. Chowdhury, and M. N. Hossain.** 2013. Identification and characterization of microorganisms: DNA-fingerprinting methods. Journal of Science and Technology **35:**397-404.
[265] **O' Sullivan, D. J.** 2000. Methods for the analysis of intestinal microflora. Current Issues in Intestinal Microbiology **1:**39-50
[266] **Holmes, B., W. R. Willcox, and S. P. Lapage.** 1978. Identification of Enterobacteriaceae by Api 20e System. Journal of Clinical Pathology **31:**22-30.
[267] **Smith, P. B., Tomfohrd.Km, A. Balows, and D. L. Rhoden.** 1972. Api System - Multitube Micromethod for Identification of Enterobacteriaceae. Applied Microbiology **24:**449-452.
[268] **Martinez-Urtaza, J., A. Lozano-Leon, A. Vina-Feas, J. Novoa, and O. Garcia-Martin.** 2006. Differences in the API 20E biochemical patterns of clinical and environmental Vibrio parahaemolyticus isolates. Fems Microbiology Letters **255:**75-81.
[269] **Southern, E. M.** 1975. Detection of Specific Sequences among DNA Fragments Separated by Gel-

Electrophoresis. Journal of Molecular Biology **98:**503-517.
[270] **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Vandelee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** 1995. Aflp - a New Technique for DNA-Fingerprinting. Nucleic Acids Research **23:**4407-4414.
[271] **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA Sequencing with Chain-Terminating Inhibitors. Proceedings of the National Academy of Sciences of the United States of America **74:**5463-5467.
[272] **Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk.** 1992. How Close Is Close - 16s Ribosomal-Rna Sequence Identity May Not Be Sufficient to Guarantee Species Identity. International Journal of Systematic Bacteriology **42:**166-170.
[273] **Stackebrandt, E. and B. M. Goebel.** 1994. A Place for DNA-DNA reassociation and 16s ribosomal-Rna sequence analysis in the present species definition in bacteriology. International Journal of Systematic Bacteriology **44:**846-849.
[274] **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumaier, M. Bachleitner, and K. H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19:**554-568.
[275] **Moreira D. and H. Philippe.** 2000. Molecular phylogeny: pitfalls and progress. International Microbiology **3:**9-16
[276] **Namsolleck, P., R. Thiel, P Lawson, K. Holstrøm, M. Rajilic, E. E. Vaughan, L. Rigottier-Gois, M. D. Collins, W. M. de Vos, and M. Blaut.** 2004. Molecular methods for the analysis of gut microbiota. Microbiology ecology in health and disease **16:**71-85.
[277] **Palys, T., L. K. Nakamura, and F. M. Cohan.** 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. International Journal of Systematic Bacteriology **47:**1145-1156.
[278] **Mollet, C., M. Drancourt, and D. Raoult.** 1997. rpoB sequence analysis as a novel basis for bacterial identification. Molecular Microbiology **26:**1005-1011.
[279] **Paradis, S., M. Boissinot, N. Paquette, S. D. Belanger, E. A. Martel, D. K. Boudreau, F. J. Picard, M. Ouellette, P. H. Roy, and M. G. Bergeron.** 2005. Phylogeny of the Enterobacteriaceae based on genes encoding elongation factor Tu and F-ATPase beta-subunit. International Journal of Systematic and Evolutionary Microbiology **55:**2013-2025.
[280] **Adekambi, T., T. M. Shinnick, D. Raoult, and M. Drancourt.** 2008. Complete *rpoB* gene sequencing as a suitable supplement to DNA-DNA hybridization for bacterial species and genus delineation. International Journal of Systematic and Evolutionary Microbiology **58:**1807-1814.
[281] **Lay, J. O.** 2001. Maldi-Tof Mass Spectrometry of Bacteria. Mass Spectrometry Reviews **20:**172-194.
[282] **Freiwald, A. and S. Sauer.** 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. Nature Protocols **4:**732-742.
[283] **Sauer, S. and M. Kliem.** 2010. Mass spectrometry tools for the classification and identification of bacteria. Nature Reviews Microbiology **8:**74-82.
[284] **Wieser, A., L. Schneider, J. T. Jung, and S. Schubert.** 2012. MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). Applied Microbiology and Biotechnology **93:**965-974.

CHAPTER II

Intestinal microbiota contribute to the endogenous formation of thiouracil in livestock

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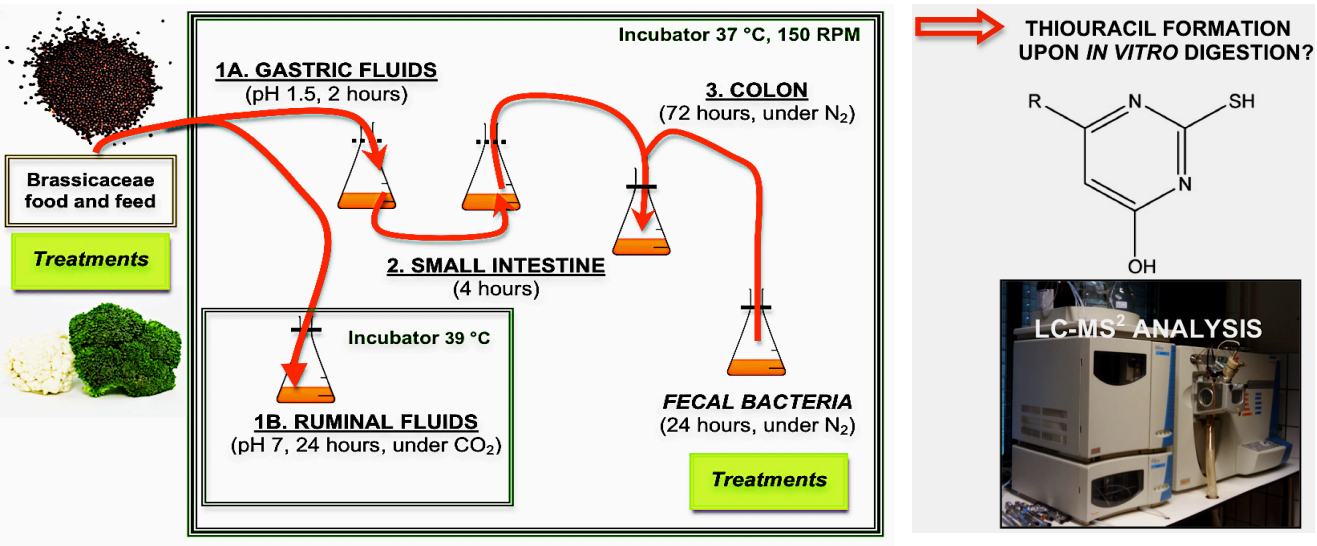
ABSTRACT

In recent years, the frequent detection of the banned substance thiouracil (TU) in livestock urine has been related to its endogenous formation following consumption of glucosinolate-rich Brassicaceae crops. Besides, TU residues have been recovered in these crops upon plant-derived myrosinase hydrolysis.

Through *in vitro* bovine and porcine static digestive simulations, the influence of gastrointestinal digestion of Brassicaceae-derived matrices on TU formation was assessed. Following derivatization and LC-MS^2 analysis TU was detected in colonic suspensions with traditional rapeseed, coarse colza '00' meal, cauliflower and broccoli ranging from 3.47 µg kg^{-1} to 30.96 µg kg^{-1} (bovine) and from 3.55 µg kg^{-1} to 26.34 µg kg^{-1} (porcine). In stomach and small intestinal fluids TU remained unfound, whereas upon rumen simulation TU was detected for coarse colza '00' meal (4.43 µg kg^{-1}) and grounded traditional rapeseed (0.35 µg kg^{-1}). The origin of this detection was investigated through filter-sterilizing and autoclaving the fecal inoculum causing a significant decrease in TU concentration, thereby reinforcing the possibility of an active bacterial involvement, which however was characterized with a high inter-animal variation. In conclusion, these results support the previously proven endogenous origin of TU and acknowledge the active role of the gastrointestinal bacteria in TU formation, through production of an extracellular component.



Keywords: *endogenous thiouracil, microbiota, Brassicaceae, in vitro static digestion simulations, LC-MS^2, livestock.*

1. INTRODUCTION

In recent years, endogenous prevalence of thiouracil (TU) has been reported in urine of livestock upon ingestion of glucosinolate-rich crops belonging to the Brassicaceae family [1]. This has been a staggering discovery, since the European Union banned the use of thyreostatic drugs in livestock for fattening purposes in 1981. Thiouracil was until recent always considered as a synthetic, orally active thyreostatic drug, which upon administration inhibits the thyroid gland by decreasing the production of thyroid hormones: thyroxin and triiodothyronine [2].

In livestock illegal TU administration pairs with a desired weight gain resulting from water retention in the digestive tract and edible tissues, which obviously degrades meat quality [3,4]. Because of the European Union banishment on thyreostatic drugs, the rightful detection of their abuse is crucial [5]. This would be the case not only for residue-analysis, but also for public health concerns, since thyreostats have been shown to exert teratogenic and carcinogenic effects, given that thiouracil is a 2b-categorized substance [6].

To allow discrimination between the illicit use of TU and possible feed related TU-levels a need for mechanistic knowledge arose. Not surprisingly, the family of the Brassicaceae was singled out as one of the most probable contributors to endogenous TU formation, as they are not only known for their high glucosinolate content, but also comprise a precursor for naturally goitrogenic substances, such as, oxazolidine-thiones and thiocyanates. These breakdown products can be naturally formed upon plant cell disruption (e.g. grounding, chewing, freezing) when the endogenous ß-thioglucosidase, myrosinase or thioglucoside glucohydrolase (E.C 3.2.1.147) is freed from the plant vacuoles, causing hydrolysis of the available glucosinolates [7-9]. As a result, several bioactive breakdown products (isothiocyanates, nitriles, thiocyanates, sulphates, epithionitriles, oxazolidine-2-thiones) are formed depending on the environmental conditions [10,11], resulting in derivates with natural thyreostatic properties [11,12].

Nowadays, growing evidence points towards the likelihood of low-level TU being endogenously formed upon digestion of the secondary plant metabolites, glucosinolates, and their degradation products [1,13]. In an attempt to confirm the possible link between glucosinolates and TU the research by Vanden Bussche *et al.* (2011) [13] demonstrated that plant-derived myrosinase hydrolysis of glucosinolate-rich Brassicaceae feed and food matrices resulted in TU detection. Thereupon the natural status of TU in the urine of various mammalians (livestock (bovine, porcine, ovine, etc.), domesticated animals (canine), and humans) was investigated. The

animals displayed traces of TU, below 10 μg L^{-1} without any diet control [14], as for humans, for whom the influence of a controlled Brassicaceae diet was investigated; TU was retrieved in 66.7% of the samples.

Besides plant-derived myrosinase hydrolysis, bacterial hydrolysis resulting from intestinal microbiota during digestion has also been linked to cause glucosinolate degradation [9,15,16]. For example, the hydrolysis of sinigrin, an aliphatic glucosinolate, was shown to be mediated by the human intestinal bacterium *Bacteroides thetaiotamicron* in gnotobiotic mice [17]. Several bacterial strains in animals have also been investigated for their glucosinolate degrading activity. For example, a *Lactobacillus* strain was identified capable of inducing a myrosinase-like enzymatic activity *in vivo* [18]. However, to the best of our knowledge no research concerning the probability of TU being formed from glucosinolates-rich Brassicaceae crops upon bacterial digestion has been performed.

Consequently, the focus of the present study was to establish the potential role of the intestinal microbiota in TU formation upon Brassicaceae digestion in animals. This was performed by conducting *in vitro* bovine and porcine gastrointestinal static digestive simulations (stomach, small intestine, large intestine). Additionally, the difference between mono- and poly-gastric animals was evaluated by incorporating digestion at the ruminal level. Various Brassicaceae feed and food matrices were evaluated by this approach.

2. MATERIALS AND METHODS

2.1. LC-MS^2 reagents and chemicals

The chemical standard 2-thiouracil (TU) was obtained from Sigma-Aldrich (St. Louis, USA). The deuterated internal standard for TU, 6-propyl-d_5-2-thiouracil (PTU-d_5)[19], was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Stock solutions of the chemical standards were prepared in methanol at a concentration of 200 ng μL^{-1}. Working solutions were diluted in methanol to 1 ng μL^{-1} for PTU-d_5 and 1 ng μL^{-1}, 0.1 ng μL^{-1} and 0.01 ng μL^{-1} for TU. Solutions were stored in dark glass recipients at 4 °C. Reagents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification steps, and of Optima® LC/MS grade for LC-MS application (Fisher Scientific UK, Loughborough, UK), respectively. The derivatization reagent, 3-iodobenzyl bromide (3-IBBr, Sigma-Aldrich, St-Louis, USA), was prepared extemporaneously (2 mg mL^{-1} methanol). The phosphate buffer, made up of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 (Merck, Darmstadt, Germany) in deionized

water, was prepared and adjusted to pH 8.

2.2. Feed and food samples

Feeds and foods affiliated to the Brassicaceae family were selected, as they are known for their high glucosinolate content. The selected representatives were traditional rapeseed (*Brassica napus* L. partim Napoleon, Institute for Agriculture and Fisheries Research (ILVO), Melle, Belgium), coarse colza '00' meal, broccoli (*Brassica oleracea* L. convar. botrytis L. var. cymosa) and cauliflower (*Brassica oleracea* L. var. botrytis L. subvar. cauliflora) (local Belgian produce). From these, Brassicaceae coarse colza '00' meal (with values of erucic acid <2% and glucosinolates <25 μmol g^{-1}) is the only allowed feed in livestock. Mostly these rapeseed meals are restricted to 30% on total feed basis due to deleterious effects of glucosinolates [13]. Additional pre-treatments (Table II.1) were applied to the feed and food matrices, consisting of various grounding and inactivation steps, resulting in 9 treatment groups in total. Grounding required the use of a mortar and for homogenization the use of a kitchen blender. In order to maintain glucosinolate concentrations, scientific reports mention limiting the water content as crucial to avoid microbial growth and prevent endogenous myrosinase activity. Storage temperatures of -20 °C or 25 °C are advised at which Brassicacea seed meals can be preserved for 30 months and presumably much longer providing they are protected from exposure to moisture conditions that promote microbial growth [20]. Therefore, all samples were ultimately freeze-dried and stored at -20 °C in plastic containers. In this way obtained TU concentrations for different feeds could be bilaterally compared based upon their dried weight concentrations.

Table II.1: Different pre-treatments applied to the Brassicaceae representatives: traditional rapeseed, coarse colza '00' meal, broccoli and cauliflower.

Treatments	**Brassicaceae foods and feeds**			
	Traditional rapeseed	**Coarse colza '00' meal**	**Broccoli**	**Cauliflower**
2 h at 100 °C	+			
5 min at 100 °C			+	+
Grounding	+			
Homogenizing		+	+	+
Freeze-drying	+	+	+	+

2.3. Preparation of buffers and broths

Digestion (stomach, small and large intestine) and fecal inoculum buffers were prepared in ultrapure water and autoclaved for 15 min, at 121 °C and 1 atm to ensure the absence of bacterial growth. Procedures following autoclavation were all performed in a laminar flow cabinet in order to exclude bacterial contamination.

Fecal inoculum buffer contained K_2HPO_4 (8.8 g L^{-1}), KH_2PO_4 (6.8 g L^{-1}) (Merck, Darmstadt, Germany) and sodium thioglycolate (1.0 g L^{-1}) (Sigma-Aldrich, Steinheim, Germany).

The stomach buffer consisted of $KHCO_3$ (10.0 g L^{-1}) (Sigma-Aldrich, Steinheim, Germany) and NaCl (5.8 g L^{-1}) (Merck, Darmstadt, Germany), which once dissolved was brought to a pH of 1.5 ± 0.1 by adding 6 M HCl (37%) (Merck, Darmstadt, Germany). A universal pH-indicator (Merck, Darmstadt, Germany) was used as an additional control. The enzyme, pepsin (0.32 g L^{-1}) (E.C. 3.4.23.2) (Sigma-Aldrich, St-Louis, USA) was filter-sterilized (Filter Millex-GV, 0.22 µm, Millex, Cork, Ireland) and added to the stomach buffer after autoclavation of the latter, in order to prevent denaturation of the enzyme.

Small intestinal buffer was made up of $NaHCO_3$ (12.6 g L^{-1}) (Merck, Darmstadt, Germany), Oxgall (6.0 g L^{-1}) (Becton, Dickson and Company, New Jersey, USA) and pancreatin (0.9 g L^{-1}) (Sigma-Aldrich, St-Louis, USA).

The ruminal phosphate buffer comprised NH_4Cl (1.4 g), $Na_2HPO_4 \cdot 12H_2O$ (28.8 g) and NaH_2PO_4 (6.2 g), which were dissolved in 1 L of distilled water with a consequent pH of 7 (Merck, Darmstadt, Germany).

The BHI (Brain Heart Infusion) broth (Oxoïd, Hampshire, England) constituted out of a ready to use powder, which had to be dissolved in ultrapure water (37 g L^{-1}). L-cysteine (0.5 g L^{-1}) (SAFC Supply Solutions, St-Louis, USA) was added for improvement of the anaerobiosis [21]. BHI broth was used to grow the fecal bacteria for 24h, which provided the needed macrobiotic culture for the large intestinal digestion. Simulator of the Human Intestinal Microbial Ecosystem broth (SHIME) was used as a medium for the actual large intestinal digestion. This medium consisted of (g L^{-1}): arabinogalactan, 1; pectin, 2; xylan, 1; mucin, 4 (all from Sigma-Aldrich, St-Louis, USA); potato starch, 3 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); glucose, 0.4 (Merck, Darmstadt, Germany); yeastcell extract, 3 (AppliChem GmbH, Darmstadt, Germany); peptone, 1 (Oxoïd, Hampshire, England); L-cystein, 0.5 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) [22]. Both SHIME and BHI broths were reboiled before use, regaining anaerobicity.

2.4. Digestion protocol

The various *in vitro* digestive simulation protocols applied during this study were adapted from scientific literature on human and animal *in vitro* models, reporting simulation of the upper and lower gastro-intestinal tract of bovines and porcines [23,24]. Several parameters (e.g. transit time, solid fluid ratios etc.) were considered in order to obtain the most optimal protocol to evaluate thiouracil formation in livestock. However, even though care has been taken to simulate the *in vivo* situation to the best possible extent, it will never be able to copy it completely and therefore *in vivo* confirmation would still be usefull in a later stage [23].

2.4.1. Fecal inoculum

In order to set up a large intestinal digestion simulation, a fecal inoculum is required. For this purpose fresh fecal matter was collected from adult female non-pregnant cows and sows from Ghent University, Faculty of Veterinary Medicine and from the Institute of Agricultural and Fisheries Research (ILVO, Melle, Belgium). The animals were housed according to animal welfare requirements, keeping them on a maintenance diet and antibiotic-free. The feces were kept at room temperature during transport before further processing was applied.

Fecal slurry was obtained by addition of 1/5 (S/L) fecal phosphate buffered saline, which subsequently was homogenized in a stomacher for 10 min. The suspension was transferred into 50 mL falcon tubes and centrifuged at 500×*g* for 2 min. To the supernatant glycerol (99.5%) (Analar Normapur, Fontenay-sous-Bois, France) was added as a cryoprotectant at a 20% (v/v) ratio and gently mixed under atmospheric conditions, before storage at -80 °C [25].

2.4.2. Ruminal inoculum

The ruminal fluid for the rumen simulation was collected before the morning feeding from three permanently cannulated cows (ILVO, Melle, Belgium) fed a hay-based diet ad libitum. Immediately after collection, samples were filtered through a metallic sieve (mesh width 1 mm) and, under CO_2 flushing, diluted five-fold with a phosphate buffer at 39 ± 1 °C. The rumen fluid and buffer mixture was kept saturated with CO_2, at 39 ± 1 °C at all times.

2.4.3. Stomach, small intestinal and large intestinal simulations

Autoclaved penicillin flasks were supplemented with feed (3 g) or food (4 g) samples following a 1/25 (S/L) ratio. The stomach digestion was simulated through addition of acidified stomach buffer (pH 1.5 ± 0.1) in a 1/10 (S/L) ratio to the samples. The flasks were capped and incubated at 37 ± 1 °C, 150 RPM for 2h. Next, 5 mL g^{-1} of pancreatic fluid was added and incubated for 4h to simulate the small intestine. Next, the fecal inoculum was thawed and diluted with reboiled BHI in a 1/9 (S/L) ratio in an autoclaved dark penicillin flask. Before incubating the flasks, anaerobic conditions were established using a N_2 flush system for 1h, at 1 bar alternating every 2 min with vacuum suction. The flasks were then incubated for 24h. This incubated bacterial inoculum was used for the large intestine digestion simulation, using 5 mL g^{-1} in combination with 5 mL g^{-1} boiled-up SHIME or BHI medium. Both were added to the small intestinal fluid flasks and flushed for 1h prior to incubation (72h) to ensure proper growing conditions for the anaerobic bacteria.

Sampling of the different digestion phases was done as secure and standardized as possible using syringes, causing as little disturbance as possible to the bacterial environment. Stomach, small intestinal and large intestinal digestion fluids (at 0, 24, 48, 72h of incubation) were gently stirred in order to homogenize before sampling.

2.4.4. Ruminal digestion simulation

The rumen digestion protocol consisted of 0.25 g or 1.0 g of freeze-dried feed, which was contained in a transparent 125 mL penicillin flask. Next, the flasks were flushed under alternating CO_2-flow and vacuum suction for anaerobiosis [26,27]. Into every flask, 25 mL of a buffer-rumen fluid mixture was added. The internal standard ethane for GC-analyses was also added (1 mL) to the flasks, which were then incubated at 39 ± 1 °C for 24h with an adapted RPM-program simulating ruminal contractions.

Subsequently, samples were put in an ice bath causing a thermal shock to the bacteria inhibiting further growth. Gases (ethane, methane, hydrogen), pH, short chain fatty acids (SCFAs) and TU were then measured. A duplo blank sample (without feed) at the beginning of the digestion and another duplo blank sample, which endured the 24h incubation, were required to assess SCFA and TU production. Combining both values allowed quantification as blank levels could be subtracted from the measured values.

2.5. Sample extraction and purification

Prior to the analysis of the digestion samples, sample preparation and clean up was needed. This procedure has been described elsewhere [28].

Briefly, 5 mL of phosphate buffer (pH 8) was added to 1 mL of digest. The internal standard (PTU-d_5) was added at 50 µg kg^{-1} followed by 100 µL of a methanolic derivatising solution containing 3-iodobenzyl bromide (2 mg mL^{-1}). Upon 10 min sonication the derivatization was allowed to proceed in the dark at 40 ± 1 °C for 1h. Afterwards the pH of the reaction mixture was adjusted to 3.6 ± 0.1 and different liquid liquid extraction steps with 3, 2 and 2 mL of diethyl ether were applied. Once evaporated to dryness under a gentle N_2 stream (2 bar, 50 ± 1°C) the samples were redissolved in 100 µL CH_2Cl_2 and 300 µL cyclohexane. Further sample clean up consisted of solid phase extraction with silica cartridges conditioned with cyclohexane and eluted with a mixture of hexane/ethyl acetate 40:60 (v/v). Again N_2 evaporation was applied. Samples were redissolved in 160 µL of 50:50 (v/v) 0.5% acetic acid and methanol.

2.6. Instrumentation

2.6.1. *Control parameters: short chain fatty acids and pH*

Colonic samples of the full digestion were assessed for their short chain fatty acid (SCFAs) content. Fatty acid extraction consisted of 2 mL sample to which 500 µL of aqueous H_2SO_4 1:1 solution (Sigma-Aldrich, St. Louis, USA), 0.4 g of NaCl (Merck, Darmstadt, Germany), 400 µL internal standard 2-methylhexane (Sigma-Aldrich, St. Louis, USA) and 2 mL diethyl ether (Merck, Darmstadt, Germany) was added. Samples were shaken for 2 min and centrifuged for 3 min at 341x*g*. Next, the supernatants were collected for GC-analyses on a Di200 (Shimadzu, 's Hertogenbosch, Netherlands) with an EC-1000 Econo-Cap capillary column (1.2 µm x 25 m x 0.53 mm) by Alltech (Laarne, Belgium). A flame ionisation detector (FID) and a Delsi Nermag 31 Integrator were used. N_2 was used as a carrier gas with a flow of 20 mL min^{-1}. Column temperature was set at 130 °C and the injector temperature at 190 °C.

The rumen samples were analyzed in the Laboratory for Animal Nutrition and Animal Product Quality of the Faculty of Bioscience Engineering (Melle, Belgium) according to the specifications mentioned in Van Ranst *et al.* (2010) [29].

The pH was measured in both colonic and ruminal samples with an electrode pH-meter (SevenEasy pH, Mettler Toledo AG, Scherzenback, Switzerland), which was calibrated before use.

2.6.2. Liquid chromatography multiple mass spectrometry (LC-MS^2)

Detection of thiouracil was achieved with a liquid chromatograph coupled to a linear ion trap mass spectrometer [13]. A Finnigan Surveyor LC-system (Thermo Electron, San Jose, USA) was combined with a Symmetry C18 column at 30 °C (5 µm x 150 mm x 2.1 mm, Waters, Milford, Massachusetts, USA) running on a 0.5% acetic acid (A) and methanol (B) 50:50 solvent combination at 0.3 mL min^{-1}. The linear gradient passed off as follows for 35 min: A/B 50:50 for 3 min, increasing to 0:100 in 17 min, and finally re-equilibrating for 10 min at 50:50. The linear ion trap mass spectrometer LTQ (Thermo Electron, San Jose, USA) was fitted with a heated electrospray ionization probe (HESI) operating in the negative ion mode and positioned on 0.5; C; 0 (front-to-back position (µm lines); probe depth (probe depth line); side-to-side position (+1 to -1 marks). Applied working conditions were as follows: source voltage at 5 kV; capillary voltage at -50 V; tube lens voltage at -128.04 V; vaporizer and capillary temperature at 250 °C and 275 °C; sheath and auxiliary gas at 30 and 5 arbitrary units (a.u.), respectively. Measured transitions are reported in Table II.2.

Table II.2: The monitored transitions by the LC-MS^2 for 2-thiouracil and its internal standard, 6-propyl-d_5-2-thiouracil.

Analyte	$[M-H]^-$	Product ions	Collision energy (eV)
2-thiouracil	343	182; 215; 309	44
6-propyl-d_5-2-thiouracil	390	127; 262; 356	30

The concentrations of TU detected in digests can be regarded as screening results and thus should be considered semi-quantitative. For further validation data we refer to *CHAPTER III* where a CC_α of 1.63 ng TU g^{-1} was reached in rapeseed digest with this method.

2.7. Quality assurance

Preceding the LC-MS^2 analysis a standard mixture of the target compound and the internal standard were injected to check the operational conditions of the LC-MS^2 device. Identification of TU was based on retention time relative to the internal standard, and the ion ratios of the product ions according to the Commission Decision 2002/657/EC [30]. Every feed or food digestion simulation was conducted in three-fold and the internal standard was added prior to clean up.

2.8. Standard addition

Quantification of TU in digestion samples was performed using the standard addition approach as described in Commission Decision 2002/657/EC [30]. Each sample was divided over 2 vials with analogous mass and volume. One aliquot, the unknown, was added with a mixture of 0.5% acetic acid and methanol (v/v) 50:50 and the other, the known, was enriched with an equal amount of analyte (thiouracil). The concentration of the addition solution was previously determined by analyzing thiouracil in digest samples and fitting these in a calibration curve in digestion buffer. Finally quantification was established through evaluation of the area ratios of the known and unknown samples using the standard addition approach[13].

2.9. Data handling

All data processing was performed with XCalibur 2.0.7 (Thermo Fisher Scientific, San Jose, CA, USA). Statistical testing (Student's-t-test, ANOVA) was carried out with Sigmaplot 12 (Systat Software GmbH, Erkrath, Germany) to assess significance (p-value < 0.05) of the type of matrix (feed and food) and the applied treatments on the recovered TU yields. Normality and equal variance were prerequisited.

3. RESULTS AND DISCUSSION

3.1. Porcine digestion

In the stomach and small intestinal fluids of the porcine simulation no thiouracil could be recovered within the methods detection limits. The probable absence or the negligible yield of TU in the stomach and the specific acidic conditions at those stages, might explain these findings. Reportedly, an *in vitro* digestion study in pigs also claimed natural goitrogens, including oxazolidine-thiones (OZTs), to fail to be detected under pepsin and small intestinal conditions [31]. The glucosinolate loss under *in vitro* peptic and small intestinal conditions in pigs ranged between 3-23% and 7-28%, respectively. Besides, the maximum activity of myrosinase occurred at moisture levels between 15% to 40% and a pH range of 3.5-8.0 [32], implying that the effect of plant enzyme myrosinase in the stomach is most probably negligible.

Analysis of colonic digestion samples however did allow TU detection (Figure II.1). From this data it may be concluded that irrespective of the pre-treatment representatives of the *Napus* group (rapeseed variants) resulted in a significantly (p-

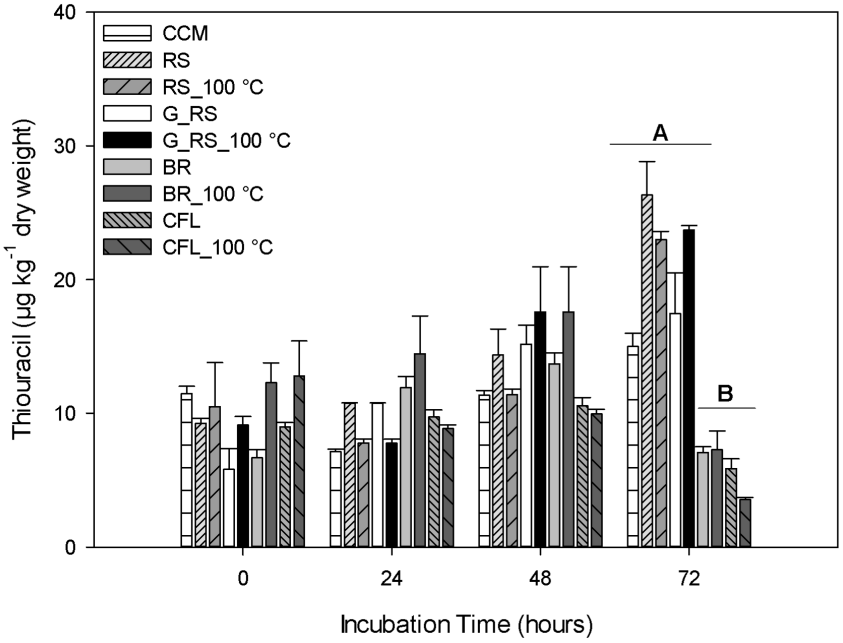


Figure II.1: Thiouracil yield (µg kg^{-1} dry matter ± standard error) of various foods and feeds in the large intestinal fluid of an *in vitro* porcine model (CCM: Coarse colza '00' meal; RS: Rapeseed; RS_100: Rapeseed, 2h at 100 °C; G_RS: Grounded Rapeseed; G_RS_100: Grounded Rapeseed, 2h at 100 °C; BR: Broccoli; BR_100: Broccoli, 5 min at 100 °C; CFL: Cauliflower; CFL_100: Cauliflower, 5 min at 100 °C). The *Napus* group (A) has a significantly (p-value < 0.05) higher TU yield than the *Oleracea* group (B) after 72h.

value < 0.05) higher TU formation compared to representatives of the *Oleracea* group (cauliflower and broccoli). This difference in TU recovery between the *Oleracea* and *Napus* matrices are with a high likelihood attributed to their difference in glucosinolate content. In parallel, evaluation of enzyme inactivation through cooking and/or grounding was performed to gain more insight in the availability and activity of the plant myrosinase. Heating at 100 °C for 2h (*Napus* group) or for 5 min (*Oleracea* group), could lead to denaturation of the plant myrosinase and the possible degradation of glucosinolates into certain by-products. It has been reported that unpressured heat treatments destroy myrosinase in for example *Brassica napus* seeds from 60 °C on and can cause glucosinolates to deteriorate and/or byproducts to evaporate [32,33]. Furthermore, grounding enables the release of glucosinolates and plant myrosinase from the feed, causing a hydrolysis reaction. This plant myrosinase has been shown to lead to TU formation in Brassicaceae feed [13].

However, grounded rapeseed showed a significant TU decrease compared to the grounded previously cooked variant. No other significant differences were found between treated and untreated Brassicaceae matrices, implying that the potential effect of these pre-treatments did not significantly contribute to the whole of the TU formation. This coincides with the previous stomach fluid results, as in analogy the applied pre-treatments also left the TU formation unaffected. Coarse colza '00' meal showed a lower TU recovery compared to rapeseed, as it consists of '00' rapeseed, which is a rapeseed variant with already a low glucosinolate content (<25 µmol g^{-1}) to start with [34]. Besides, coarse colza '00' also undergoes grounding, hot pressing (80-140 °C), hexane extraction and toasting during its production process, which may negatively influence further TU formation upon digestion.

During digestion, parameters including pH and volatile fatty acids were used to evaluate the proper digestion conditions. Short chain fatty acid recoveries in the *in vitro* large intestinal fluids approximated the known *in vitro* acetate > propionate > butyrate ratio, which *in vivo* approaches a 70 : 20 : 10 ratio in livestock [35,36]. Both volatile fatty acids and pH values were found within the physiological values for bacterial growth, which are substrate, species and animal dependent, as described elsewhere [35,37].

In general, for the porcine model it may be concluded that the large intestine is the main site of TU formation. In an ulterior research [13] identical Brassicaceae feeds and foods upon plant-myrosinase hydrolysis were analyzed for their TU content. In summary, TU was retrieved in the highest concentrations for broccoli, traditional rapeseed and coarse colza '00' meal displayed (5.98 µg kg^{-1}, 1.45 µg kg^{-1} and 1.59 µg kg^{-1}, based on dry weight). Other samples yielded very low TU concentrations (<1.0 µg kg^{-1}) (cauliflower, rapeseed cake) or undetectable signals (feeding cabbage, feed 30% rapeseed '00') due to background noise. When comparing these absolute concentrations, the here obtained TU concentrations upon digestion were up to a factor 10 higher depending on the matrix. This confirms the involvement of colonic digestion and most likely, microbial fermentation in the formation of TU.

3.2. Bovine digestion

3.2.1. *Rumen digestion*

Reportedly ruminants are more tolerant to glucosinolate fermentation breakdown products at the ruminal level compared to other animals consuming forage brassica crops [38]. On that account, the capacity of ruminal microbiota to form TU at the ruminal level upon brassicaceae digestion was evaluated. Glucosinolate

fermentation was assessed through a static 24h *in vitro* rumen digestion with coarse colza '00' meal, whole rapeseed and grounded rapeseed fermentation in triplicate. But only when feed amounts were increased from 0.25 g dry weight (S/L = 1/100) to 1 g dry weight (S/L = 1/25), thereby matching the S/L ratio of the intestinal digestion simulations, TU was detected in the rumen. Coarse colza '00' meal showed a TU yield of 4.43 ± 0.35 µg kg^{-1} dry matter and grounded traditional rapeseed of 0.35 ± 0.01 µg kg^{-1} dry matter. For whole rapeseed TU remained unfound in the rumen, most probably due to the low availability of this substrate to the ruminal bacteria [39,40].
Because increasing the feed amount might saturate the ruminal digestion system by increasing the organic matter in spite of the ruminal buffer mixture, the pH, produced gases and short chain fatty acids (SCFAs) were carefully analyzed for abnormalities [41,42]. All measured values approximated the normal physiological conditions required for ruminal fermentation, i.e. pH ranging from 6.0 to 6.8 and SCFA proportions within the expected ratios (i.e. acetate : propionate : butyrate between 70 : 20 : 10 and 60 : 30 : 10) [42].
Therefore, these results demonstrate the TU forming potential of the rumen microbiota under specific *in vitro* conditions, at relatively low concentrations (0.35 and 4.42 µg kg^{-1} dry matter) as compared to the TU yields observed in the bovine colonic simulation for coarse colza '00' meal and traditional rapeseed, respectively 7.86 and 30.96 µg kg^{-1} dry matter.

3.2.2. Bovine colonic digestion

Similar results as to the ones obtained in the porcine model appeared when evaluating the bovine colonic digestive suspension for TU (Figure II.2). In summary, the *Napus* group showed a significantly higher TU forming capacity compared to the *Oleracea* group, with coarse colza '00' meal exerting a significantly low TU recovery compared to the other traditional rapeseed treatment groups.
The bovine model seemed equally unaffected by the inactivation treatments as even less significance (p-value > 0.05) was found between treated and untreated matrices then for the porcine model. Therefore, the observed TU yield variation is most probably due to variation in the microbial colonic population of these two species.

The pH, with a reported *in vivo* median value of 7.4 [37], and short chain fatty acids (SCFAs), with a reported 70 : 20 : 10 ratio [36] were also observed in the bovine model. All obtained values were found within the excepted physiological ranges for digestion and bacterial growth [35,37].

In general, for bovines the large intestine may, in line with porcines, be considered as the main site of TU formation, with a, respectively, minimal influence designated to the rumen.

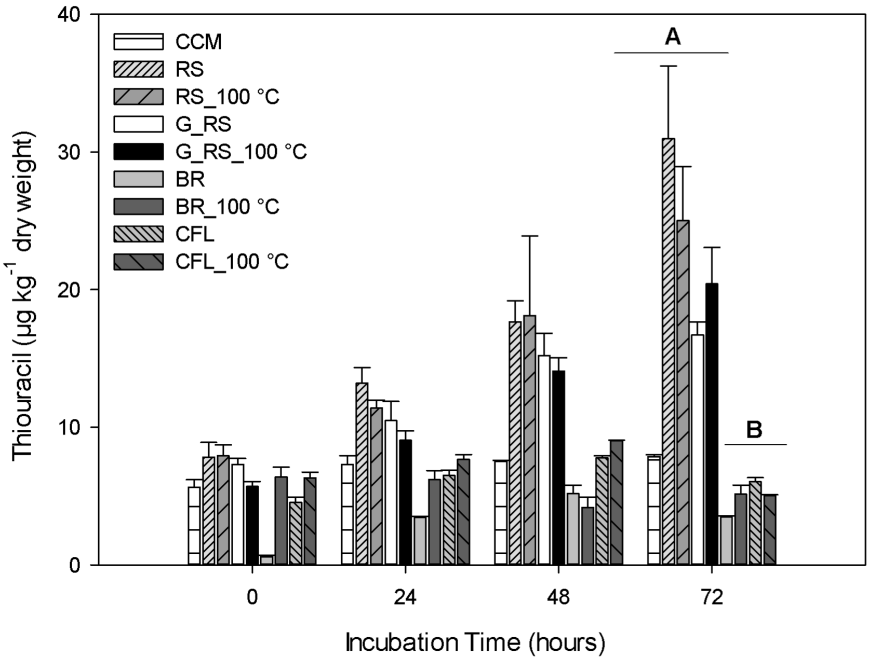


Figure II.2: Thiouracil yield (µg kg^{-1} dry matter ± standard error) of various foods and feeds in the large intestinal fluid of the in vitro bovine model (CCM: Coarse colza '00' meal; RS: Rapeseed; RS_100: Rapeseed, 2h at 100 °C; G_RS: Grounded Rapeseed; G_RS_100: Grounded Rapeseed, 2h at 100 °C; BR: Broccoli; BR_100: Broccoli, 5 min at 100 °C; CFL: Cauliflower; CFL_100: Cauliflower, 5 min at 100 °C). The *Napus* group (A) has a significantly (p-value < 0.05) higher TU yield than the *Oleracea* group (B) after 72h.

3.3. Uncovering the mechanism of TU formation in the colon

For both the porcine and bovine model, TU formation was only observed in the highly microbially populated areas of the digestive tract, i.e. the large intestine (both for the porcine and bovine model) and to a lesser extent in the bovine rumen. Therefore, the assumption was made that gastric and small intestinal digestions do not significantly (p-value > 0.05) alter the ultimate large intestinal TU concentration. Additional experiments were designed to further investigate the nature of TU formation in the large intestine.

Up till now the equilibrated SHIME medium had been used, which is specifically designed to maintain and not immediately boost the intestinal microbiota [22]. Therefore another broth was chosen to significantly enhance bacterial growth. In this case Brain Heart Infusion (BHI) broth, which is a liquid medium, very rich in nutrients and suitable for the cultivation of a broad range of bacteria, was the recommended choice [43]. In the presence of BHI broth traditional rapeseed (RS) and coarse colza '00' meal (CCM), as the highest and lowest TU producing feedstuffs from previous experiments, were incubated with the porcine and bovine inocula for a single-step large intestinal simulation. Frequent sampling (1, 3, 6, 24, 48, 72 h) allowed insight in the timescale of TU transformation throughout digestion for both species. Subsequently, a tipping point in TU formation was revealed for traditional rapeseed at 24 h for the bovine model and at 48 h for the porcine model (Figure II.3).

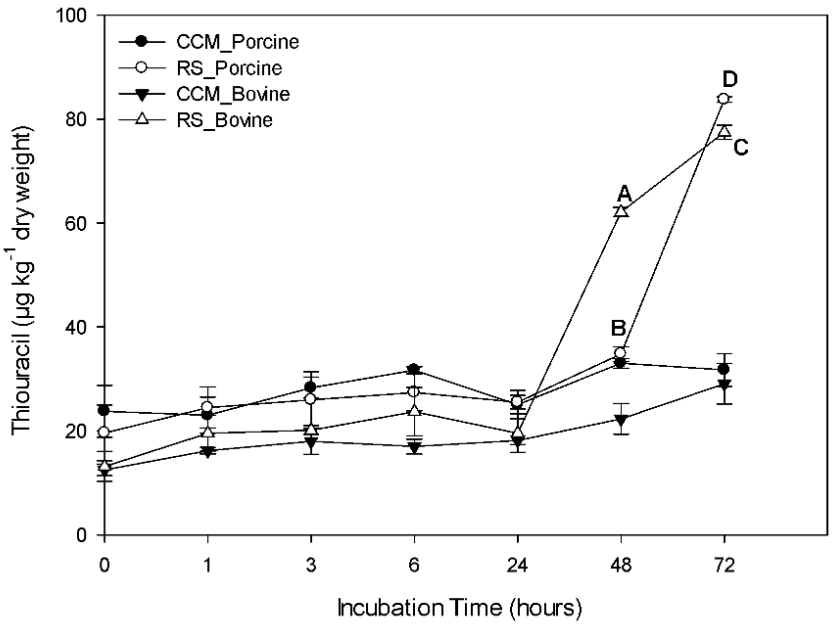


Figure II.3: Representation of the TU production (µg kg^{-1} dry matter ± standard error) upon digestion of coarse colza '00' meal and traditional rapeseed in the bovine and porcine sole large intestine digestion. A demonstrates a significant (p-value < 0.05) increase of TU at 48h of incubation compared to B, whereas at 72h C shows a slight, but significant, TU decrease compared to D.

Coarse colza '00' meal on the other hand showed a steady, but slow increase in TU concentration. The TU concentrations obtained through this experimental set-up were twice as high as compared to the previous full digestion approach, confirming the positive influence of the BHI medium on bacterial growth and TU formation. The

difference in tipping point between the porcine and bovine model is most likely influenced by interspecies microbial diversity and the subsequent species-specific fecal metabolite profile [44,45]. Human, primate and non-primate mammalian fecal microbial populations have been shown to be more similar to each other within the same species than between different host species [44]. Besides, bacterial diversity increases based upon phylogeny from carnivory to omnivory to herbivory, which might explain the variations we see between the bovine and porcine model, as one is omnivore and the other herbivore. Furthermore, the digestive bacterial population is subject to diet and therefore forced to co-diversify with their hosts eating behavior [44].

Thereupon, inter-animal variability was evaluated within one species (porcine), through comparison of the fecal inocula of 10 female porcines upon rapeseed colonic digestion. Eight animals originated (2-6; 8-10) from the Institute for Agriculture and Fisheries Research (Melle, Belgium) and two animals (1, 7) from the Faculty of Veterinary Medicine (Ghent, Belgium), both individual groups comprised animals of the same age, sex and were held under equal housing and feeding conditions (Figure II.4).

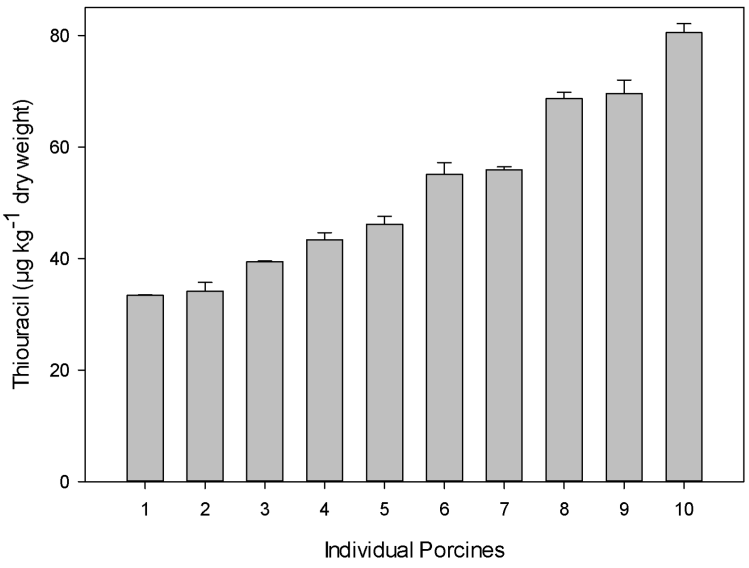


Figure II.4: Inter-animal variation in porcines of TU abundance (µg kg^{-1} dry matter ± standard error) upon a sole colonic simulation of the same feed (rapeseed).

TU production showed a significant (p-value < 0.05) inter-animal variation between the porcines even when they were kept under similar housing conditions. The coefficient of variance (CV%) of the TU concentrations at the beginning and the end of the 72h incubation amounted at 73% and 31%, respectively. These findings support

the bacterial involvement in TU formation, since the inter-animal differences in colonic bacterial composition and activities most probably are responsible for the observed differences in TU yield between various pigs. Microbial digestion of feed has previously been reported in literature to engender a high inter-animal variation in pigs [46].
Subsequently, confirmation of the mechanistic nature of the microbial formation of TU was envisaged and to this extent, the influence of autoclavation (121 °C, 1 atm, 15 min) and filter-sterilization of the inoculum (0.22 µm), as well as, autoclavation of the rapeseed feed source (121 °C, 1 atm, 15 min) on TU detection was evaluated (n=3). The highest TU-producing porcine inoculum was subjected to different treatments in a colonic *in vitro* digestion and compared to a positive control.
Autoclavation showed no significant TU formation, whereas TU formation with the filter-sterilized inoculum and autoclaved feed were left unaffected as compared to the control (Figure II.5).

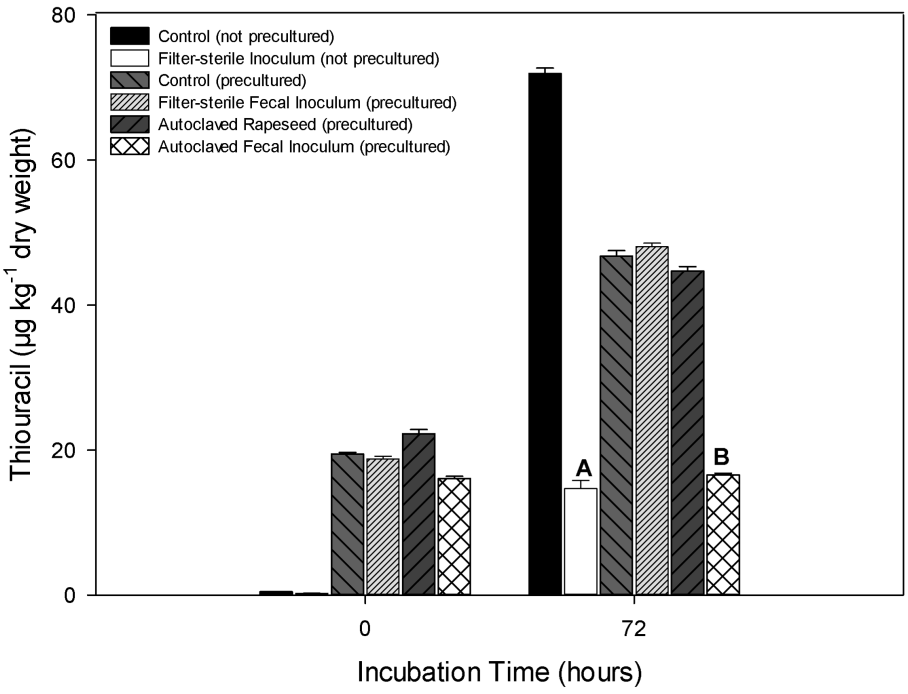


Figure II.5: Representation of the effects of preculturation, autoclavation and filter-sterilization on TU formation (µg kg^{-1} dry matter ± standard error) during a porcine colonic rapeseed digestion simulation. Treatments A and B demonstrate significant (p-value < 0.05) TU lowering effect.

This indicates that bacterial contamination of feed does not play a role in the conversion of Brassicaceae feed precursors to TU. Since filter-sterilization removes all

bacteria from the inoculum and still causes TU formation concurring with the positive control, the involvement of a bacterially produced extracellular secondary product, such as an enzyme involved in TU formation, may be presumed. In order to further justify this presumption another set-up compared a filter-sterilized inoculum to a positive control sample (n=3). In this particular case the inoculum was not pre-enriched for 24h prior to the 72h incubation, which was the case for all previous digestions. Thereby, the effect of filter-sterilization (0.22 µm) on TU formation could be evaluated, since bacteria were removed from the beginning of the incubation. After 72h of incubation the filter-sterilized inoculum was found to be 80% lower in TU concentration compared to the positive control. Based on these results attempts were made to evaluate whether this responsible enzyme could be the hypothesized bacterial myrosinase, alias ß-thioglucosidase or thioglucoside glucohydrolase (E.C 3.2.1.147). To this end, the synthetically available glucosinolate, sinigrin, was added to colonic digestive fluids. If myrosinase was present in these suspensions, this would lead to the hydrolysis of sinigrin with loss of glucose, which then could be detected by an HPLC-ELSD system [13]. To this point we have been unsuccessful in achieving this conversion based upon 'bacterial myrosinase' in the digesta. But, when adding plant myrosinase extracted from *Sinapis alba* L. seeds (white mustard) to these sinigrin enriched digesta, glucose was detected, implying that the conversion is indeed possible within this matrix. However, literature reports cell-free supernatants of *Lactobacillus agilis* cultures to be incapable of sinigrin degradation [47] and implies a cell-associated activity for 'bacterial myrosinase'. This cannot be correlated with our findings, as filter-sterilized precultured digesta were able to form TU in the absence of bacterial cells. Furthermore, when estimating the relative myrosinase amount, which could have been present in the digesta, based upon their TU yield, the calculated amount of bacterial myrosinase should have approximately ranged between 10 and 30 times the amount of myrosinase administered in the research by Vanden Bussche *et al.* (2011) [13] for recovery of the same TU amounts in identical feed matrices. Therefore, if bacterial myrosinase had been formed in the digestion fluids sinigrin degradation should have taken place, subsequently causing glucose detection. These discrepancies indicate the likelihood that TU formation upon Brassicaceae digestion is independent of a myrosinase-like bacterial enzyme, but involves an unidentified bacterial enzyme or metabolite.

In conclusion, the results obtained from this study strongly imply bacteria to be involved in the gastrointestinal formation and detection of TU in livestock. Brassicaceae digestion in bovine and porcine *in vitro* simulations showed detectable

TU levels in the large intestine for both models and in the rumen for bovines, although to a considerable lower extent. Besides, the influence of plant myrosinase was found negligible as inactivation treatments failed to significantly coherently decrease TU yields. On the other hand the influence of the bacterial population was quite important, as different TU yields were recovered for the same Brassicaceae feed within the same species. The bovine and porcine culture also reacted differently for some feeds. The agent responsible for the TU formation upon digestion of Brassicaceae matrices is likely to be a thermosensitive component with a cell-independent activity, which resides extracellularly after bacterial formation through a fortuitous process, which is independent of Brassicaceae presence. Further investigation regarding this mediator involved in TU formation upon Brassicaceae digestion in livestock seems desirable.

4. ACKNOWLEDGEMENTS

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5. REFERENCES

[1] **Pinel, G., S. Mathieu, N. Cesbron, D. Maume, H. F. De Brabander, F. Andre, and B. Le Bizec.** 2006. Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyrostats in meat production. Food Additives and Contaminants **23:**974-980.

[2] **Courtheyn, D., B. Le Bizec, G. Brambilla, H. F. De Brabander, E. Cobbaert, A. V. de Wiele, J. Vercammen, and K. De Wasch.** 2002. Recent developments in the use and abuse of growth promoters. Analytica Chimica Acta **473:**71-82.

[3] **Kotter, L., G. Terplan, and J. Schulz.** 1959. Biological demonstration of inhibitors in foodstuff of animal origin. Archiv für lebensmittelhygiene **10:**145-152.

[4] **Derblom, H., H. Johansson, and G. Nylander.** 1963. Thyroid hormone activity and gastrointestinal function, an experimental study in rats. Acta Chirurgica Scandinavica **10:**1-5.

[5] **European Community.** 1981. Council Directive 81/602/EC. Official Journal of the European Communities **L 222:**32-33.

[6] **International Agency for Research on Cancer.** 2011. IARC monographs on the evaluation of carcinogenic risks to humans. Available from: http://monographs.iarc.fr/ENG/Classification/index.php.

[7] **Verkerk, R., M. S. vanderGaag, M. Dekker, and W. M. F. Jongen.** 1997. Effects of processing conditions on glucosinolates in cruciferous vegetables. Cancer Letters **114:**193-194.

[8] **Kjaer, A.** 1960. Naturally derived isothiocyanate (mustard oil) and their parent glucosides. In Progress in the Chemistry of Organic Natural Products; Hetz, W., Grisebach, H., Kirby, G. W., Eds.; Springer-Verlag: Berlin, Germany, pp 122-129.

[9] **Tripathi, M. K., and A. S. Mishra.** 2007. Glucosinolates in animal nutrition: A review. Animal Feed Science and Technology **132:**1-27.

[10] **Rosa, E. A. S., and A. S. Rodrigues.** 2001. Total and individual glucosinolate content in 11 broccoli cultivars grown in early and late seasons. Hortscience **36:**56-59.

[11] **Bones, A. M., and J. T. Rossiter.** 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. Physiologia Plantarum **97:**194-208.

[12] **Halkier, B. A., and L. C. Du.** 1997. The biosynthesis of glucosinolates. Trends in Plant Science **2:**425-431.

[13] **Vanden Bussche, J., J. A. L. Kiebooms, N. De Clercq, Y. Deceuninck, B. Le Bizec, H. F. De Brabander, and L. Vanhaecke.** 2011. Feed or food responsible for the presence of low-level thiouracil in urine of livestock and humans? Journal of Agricultural and Food Chemistry **59:**5786-5792.

[14] **Vanden Bussche, J., L. Vanhaecke, Y. Deceuninck, K. Wille, K. Bekaert, B. Le Bizec, and H. F. De Brabander.** 2011. Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry detection of naturally occuring thiouracil in urine of untreated livestock, domesticated animals and humans. Food Additives and Contaminants **28:**166-172.

[15] **Krul, C., C. Humblot, C. Philippe, M. Vermeulen, M. van Nuenen, R. Havenaar, and S. Rabot.** 2002. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic in vitro large-intestinal model. Carcinogenesis **23:**1009-1016.

[16] **Cheng, D. L., K. Hashimoto, and Y. Uda.** 2004. In vitro digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. Food and Chemical Toxicology **42:**351-357.

[17] **Elfoul, L., S. Rabot, N. Khelifa, A. Quinsac, A. Duguay, and A. Rimbault.** 2001. Formation of allyl isothiocyanate from sinigrin in the digestive tract of rats monoassociated with a human colonic strain of *Bacteroides thetaiotaomicron*. Fems Microbiology Letters **197:**99-103.

[18] **Nugon-baudon, L., S. Rabot, J. M. Wal, and O. Szylit.** 1990. Interactions of the intestinal microflora with glucosinolates in rapeseed meal toxicity - 1st Evidence of an Intestinal Lactobacillus possessing a myrosinase-like activity *in vivo*. Journal of the Science of Food and Agriculture **52:**547-559.

[19] **Lõhmus, M., K. Kallaste, and B. Le Bizec.** 2009. Determination of thyreostats in urine and thyroid gland by ultra high performance liquid chromatography tandem mass spectrometry. Journal of Chromatography A **1216:**8080-8089.

[20] **Morra, M. J., and V. Borek.** 2010. Glucosinolate preservation in stored Brassicaceae seed meals. Journal of Stored Products Research **46:**98-102.

[21] **Shanson, D. C., and J. Singh.** 1981. Effect of adding cysteine to brain-heart infusion broth on the isolation of *Bacteroides fragilis* from experimental blood cultures. J Clin Pathol **34:**221-223.

[22] **Molly, K., M. Vandewoestyne, I. Desmet, and W. Verstraete.** 1994. Validation of the Simulator of the Human Intestinal Microbial Ecosystem (Shime) reactor using microorganism-associated activities. Microbial Ecology in Health and Disease **7:**191-200.

[23] **Boisen, S. and J. A. Fernandez.** 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. Animal Feed Science and Technology **68:**277-286.

[24] **Gawlik-Dziki, U., D. Dziki, B. Baraniak, and R. F. Lin.** 2009. The effect of simulated digestion *in vitro* on bioactivity of wheat bread with Tartary buckwheat flavones addition. Lwt-Food Science and Technology **42:**137-143.

[25] **Vanhaecke, L., N. Van Hoof, W. Van Brabandt, B. Soenen, A. Heyerick, N. De Kimpe, D. De Keukeleire, W. Verstraete, and T. Van de Wiele.** 2006. Metabolism of the food-associated carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine by human intestinal microbiota. Journal of Agricultural and Food Chemistry **54:**3454-3461.
[26] **Boeckaert, C., B. Vlaeminck, J. Mestdagh, and V. Fievez.** 2007. *In vitro* examination of DHA-edible micro algae 1. Effect on rumen lipolysis and biohydrogenation of linoleic and linolenic acids. Animal Feed Science and Technology **136:**63-79.
[27] **Fievez, V., F. Dohme, M. Danneels, K. Raes, and D. Demeyer.** 2003. Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation *in vitro* and *in vivo*. Animal Feed Science and Technology **104:**41-58.
[28] **Pinel, G., E. Bichon, K. Pouponneau, D. Maume, F. Andre, and B. Le Bizec.** 2005. Multi-residue method for the determination of thyreostats in urine samples using liquid chromatography coupled to tandem mass spectrometry after derivatisation with 3-iodobenzylbromide. Journal of Chromatography A **1085:**247-252.
[29] **Van Ranst, G., V. Fievez, M. Vandewalle, C. Van Waes, J. De Riek and E. Van Bockstaele.** 2010. Influence of damaging and wilting red clover on lipid metabolism during ensiling and *in vitro* rumen incubation. Animal **4:**1528-1540.
[30] **European Community.** 2002. Commission Decision 2002/657/EC. Official Journal of the European Communities **L 221:**8-36.
[31] **Maskell, I. and R. Smithard.** 1994. Degradation of glucosinolates during *in vitro* incubations of rapeseed meal with myrosinase *(EC*3.2.3 1) and with pepsin *(EC* 3.4.23.l)-hydrochloric acid, and contents of porcine small intestine and caecum. British Journal of Nutrition **72:**455-466.
[32] **Tripathi, M. K., I. S. Agrawal, S. D. Sharma, and D. P. Mishra.** 2001. Effect of untreated, HCl treated or copper and iodine supplemented high glucosinolate mustard (*Brassica juncea*) meal on nutrient utilization, liver enzymes, thyroid hormones and growth of calves. Animal Feed Science and Technology **92:**73-85.
[33] **Van Eylen, D., Indrawati, M. Hendrickx, and A. Van Loey.** 2006. Temperature and pressure stability of mustard seed (*Sinapis alba L.*) myrosinase. Food Chemistry **97:**263-271.
[34] **European Community.** 2003. Commission Regulation (EC) No. 1035/2003. Official Journal of the European Communities **L 150:**351-357.
[35] **Coles, L. T., P. J. Moughan, and A. J. Darragh.** 2005. *In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. Animal Feed Science and Technology **123:**421-444.
[36] **Bergman, E. N.** 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews **70:**567-590.
[37] **Kararli, T. T.** 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of Humans and commonly used laboratory animals. Biopharmaceutics & Drug Disposition **16:**351-380.
[38] **Duncan, A. J., and J. A. Milne.** 1992. Rumen microbial degradation of allyl cyanide as a possible explanation for the tolerance of sheep to *Brassica*-derived glucosinolates. Journal of the Science of Food and Agriculture **58:**15-19.
[39] **Nordin, M., and R. C. Campling.** 1976. Digestibility studies with cows Given whole and rolled cereal grains. Animal Production **23:**305-315.
[40] **Murphy, J. J., and F. Omara.** 1993. Nutritional manipulation of milk protein concentration and Its impact on the dairy industry. Livestock Production Science **35:**117-134.
[41] **Lee, S. S., M. B. Chang, and J. K. Ha.** 2004. Effects of bacterial fraction and proportion of silage and concentrate on rumen fermentation and gas production profile. Asian-Australasian Journal of Animal Sciences **17:**643-647.
[42] **Sjaastad, Ø. V., K. Hove, and O. Sand.** 2004. In Physiology of the Domestic Animals, The Digestive system, 2nd edition, C. Steel, ed., Scandinavian Veterinary Press, Oslo, Finland, pp 490-563.
[43] **Thermo Fisher Scientific, Oxoid Ltd.** Dehydrated Culture Media: Brain Heart Infusion broth. Available from: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM1135&org=133&c=UK&lang=EN)
[44] **Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon.** 2008. Evolution of mammals and their gut microbes. Science **320:**1647-1651.
[45] **Saric, J, Y. Wang, J. Li, M. Coen, J. Utzinger, J. R. Marchesi, J. Keiser, K. Veselkov, J. C. Lindon, J. K. Nicholson, and E. Holmes.** 2008. Species variation in the fecal metabolome gives insight into differential gastrointestinal function. Journal of Proteome Research **7:** 352-360.
[46] **Jensen, M. T., R. P. Cox, and B. B. Jensen.** 1995. Microbial-Production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat. Animal Science **61:**293-304.
[47] **Palop, M. L., J. P. Smiths, and B. Tenbrink.** 1995. Degradation of sinigrin by *Lactobacillus agilis* strain R16. International Journal of Food Microbiology **26:**219-229.

CHAPTER III

Identification and characterisation of thiouracil forming bacteria upon porcine *in vitro* digestion of Brassicaceae feed

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ABSTRACT

In recent years, the frequent detection of the banned thyreostat, thiouracil (TU), in livestock urine has been related to endogenous TU formation following digestion of glucosinolate-rich Brassicaceae crops. Recently, it was demonstrated that fecal bacteria upon *in vitro* digestion of Brassicaceae, induce TU detection in livestock (porcine livestock > bovines). Therefore, the present study intended to isolate and identify bacteria involved in this intestinal TU formation upon Brassicaceae digestion, and gain more insight in the underlying mechanism in porcines. Twenty porcine fecal inocula (gilts-multiparous sows) were assessed through static *in vitro* colonic digestion simulations with rapeseed. After derivatisation and extraction of the fecal suspensions, TU was analysed using LC-MS^2. On average lower TU concentrations were observed in fecal colonic simulations in gilts (8.35 ± 3.42 SD ng g^{-1} rapeseed) in comparison to multiparous sows (52.63 ng g^{-1} ± 16.17 SD), which correlates with maturation of the gut microbial population with age. Further exploration of the mechanism, showed cell-dependent activity of the microbial conversion and sustained TU-forming activity after subjection of the fecal inoculum to moderate heat over a time-span of up to 30 minutes. Finally, nine TU producing bacteria were successfully isolated and identified by a combination of biochemical and molecular techniques as *Escherichia coli (n=5), Lactobacillus reuteri (n=2), Enterococcus faecium (n=1), Salmonella enterica ssp. arizonae (n=1)*. This study demonstrates that endogenous formation of TU is Brassicaceae-induced and occurs under colonic conditions through a most likely myrosinase-like enzyme activity expressed by different common intestinal bacterial species.

Keywords: endogenous thiouracil, rapeseed, static in vitro colonic digestion simulations, LC-MS^2, DNA-sequencing, pig.

1. INTRODUCTION

The continuous challenge of maintaining a safe and healthy food chain has lead to some remarkable findings regarding the occurrence of controlled or forbidden residues in animal husbandry. Generally, it was believed that the detection of banned substances in livestock could be attributed to fraudulous administration. But, in the last decade, this belief has been disputed by recurring discrepancies showing that certain forbidden substances, such as the thyreostat thiouracil (TU), may occur in animal matrices without a history of illicit administration [1,2]. This facilitates the incidence of possible false accusations of illicit use due to non-compliant TU results during national control plan campaigns, because in this case in particular no differentiation between exogenous administration and endogenous formation is yet routinely applicable. Thiouracil (TU), a thyreostatic drug of synthetic origin, has been forbidden [3] in animal production by the European Union more then three decades ago. The equivocal reason behind this decision was on one hand, the fraudulent slaughtering weight gain, which could be achieved through TU administration causing water retention in edible tissues and gut, inevitably deteriorating meat quality. On the other hand, the potential hazard for public health safety was taken into account since carcinogenic [4] and teratogenic effects had been described [5,6,7]. Consequently, the European Union demands from its member states to perform thyreostat analysis on diverse matrices (e.g. urine, meat). As a guideline a European Minimum Required Performance Limit (MRPL) was stipulated for the analytical detection of thyreostats, insuring detection of 100 µg L^{-1} or kg^{-1} in all matrices [8]. In recent years, analytical detection methods are capable to reach detection limits below 10 µg L^{-1} [9,10, 11]. As a result, low-level TU concentrations (< 10 µg L^{-1}) were frequently detected in national control plans in urine of untreated livestock [12,13,2]. Because of this, the European Union Reference Laboratory set out a recommended concentration (RC) of 10 µg L^{-1} or kg^{-1} (urine and thyroid) for thiouracil [14], which may be considered as a technical guideline for laboratories involved in EU control plans, but has no legal constraining power.

Pinel *et al.* [1] were the first to describe the possibility of a feed-related origin of TU in livestock. More specifically they discovered that digestion of Brassicaceae crops caused detection of TU (< 10 µg L^{-1}) in bovine urine. Brassicaceae crops (e.g. rapeseed, cabbage) have been widely used in livestock feedstuffs for their cheap and high quality nitrogen content, especially during winter. Another well-known characteristic is however their high glucosinolate content [15]. Upon plant disruption

(e.g. chewing), glucosinolates, located in the idioblast cells of the plant, are freed from their vacuoles together with the plant-derived enzyme myrosinase (ß-thioglucosidase, E.C 3.2.1.147), which is released from the parenchymatous tissue [15], causing various breakdown products (oxazolidine-thiones, nitriles, epithionitriles thiocyanates, isothiocyanates and thiourea) depending on pH [16,17]. Some of these breakdown products are natural goitrogenic substances affecting the thyroid function, e.g.: oxazolidine-thiones (goitrin), thiocyanates and thiourea [18]. Negative effects of glucosinolates on animal production are proportional to their dietary concentration [19] and the exploitability of glucosinolate-rich crops in the feed industry is consequently limited [15]. Subsequently, this has led to a minimisation of glucosinolate content of Brassicaceae crops with the development of '00' - type plants, which have a low glucosinolate content (max. of 25 µmol g^{-1} at a moisture content of 9%) and erucic acid content (< 2% of the total fatty acid content) [20].

In order to confirm the possible link between glucosinolates and TU formation, the research by Vanden Bussche *et al.* [21] demonstrated that the interference of plant-derived myrosinase was a necessity for the (*in vitro*) hydrolysis of glucosinolate-rich Brassicaceae food and feed matrices into TU. Additionally, the natural occurrence of TU in the urine of various domesticated animals, and humans was investigated. The animals displayed traces of TU, below 10 µg L^{-1} without any diet control [2], as for humans, for whom the influence of a controlled Brassicaceae diet was investigated, low-level TU was retrieved in 66.7% of the samples.

Besides plant-derived myrosinase hydrolysis, bacterial hydrolysis resulting from intestinal microbiota has also been linked to cause glucosinolate degradation during digestion [22,23,24]. For example, the hydrolysis of sinigrin, an aliphatic glucosinolate, can be mediated by the human intestinal bacterium *Bacteroides thetaiotamicron* in gnotobiotic mice [25]. Several bacterial strains in animals have also been investigated for their glucosinolate degrading activity. For example, a *Lactobacillus* strain was capable of inducing a myrosinase-like enzymatic activity *in vivo* [26] and the *Lactobacillus agilis* R16 strain could degrade sinigrin and glucosinolates in brown mustard seed extracts [27]. The study by Kiebooms *et al*. [28] was the first to demonstrate *in vitro* that TU formation in bovines and porcines was influenced by a bacterial component and identified the large intestine, with its high microbial number and diversity [29,30], as the main transformation site. Concentrations up to 80 µg kg^{-1} TU were registered in *in vitro* livestock digestion simulations with Brassicaceae.

Consequently, the focus of the present study was to further inquire the potential and identity of the intestinal microbiota as well as the mechanisms involved in TU formation upon Brassicaceae digestion in livestock by conducting static *in vitro*

colonic digestion simulations in porcines, because of their high TU producing capacity [28].

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The chemical standard 2-thiouracil (TU) was obtained from Sigma-Aldrich (St. Louis, USA). The deuterated internal standard for TU, 6-propyl-d_5-2-thiouracil (PTU-d_5), was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Stock solutions of the chemical standards were prepared in methanol at a concentration of 200 ng μL^{-1}. Working solutions were diluted in methanol to 1 ng μL^{-1} for PTU-d_5 and 1 ng μL^{-1}, 0.1 ng μL^{-1} and 0.01 ng μL^{-1} for TU. Solutions were stored in dark glass recipients at 4 °C. Reagents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification purposes, and of Optima® LC-MS grade for LC-MS application (Fisher Scientific UK, Loughborough, UK), respectively. The derivatisation reagent, 3-iodobenzyl bromide (3-IBBr, Sigma-Aldrich, St-Louis, USA), was prepared extemporaneously (2 mg mL^{-1} methanol). A phosphate buffer, consisting of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 (Merck, Darmstadt, Germany) in deionized water, was prepared and adjusted to pH 8 with 6 M HCl.

2.2. Feed and faeces sampling

Traditional rapeseed (*Brassica napus* L. partim Napoleon, Institute for Agriculture and Fisheries Research (ILVO), Melle, Belgium) was used for the *in vitro* digestion experiments as a substrate for high TU production upon bacterial fermentation. The large intestinal *in vitro* digestion simulations required sampling of several porcine fecal inocula (FI). For this purpose fresh fecal matter was collected upon defecation from 10 female gilts (< 8 months old) waiting for their first insemination [31] and 10 adult multiparous female porcines from the Institute of Agricultural and Fisheries Research (ILVO, Melle, Belgium) and Ghent University, Faculty of Veterinary Medicine (Ghent, Belgium). The animals were housed according to animal welfare requirements, keeping them on a standard antibiotic-free maintenance diet sustaining the general metabolism. The faeces aliquots were quickly transported at room temperature to the lab where further processing was performed.

2.3. *In vitro* large intestinal digestion

2.3.1. Preparation of buffer, broth and agar

Digestion broth media, agar media and phosphate buffered saline were prepared in ultrapure water (UPW), processed conform to the manufacturing guidelines (Sartorius stedim Biotech GmbH, Göttingen, Germany) and autoclaved for 15 minutes, at 121 °C and 1 atm to ensure the absence of bacterial contamination and subsequent growth. Any procedure following autoclavation was performed in a laminar flow cabinet to prevent bacterial contamination. Autoclaved digestion broths, buffers and agar plates were all stored tightly sealed at 4 °C no longer than 1 month.

Phosphate buffered saline, wherein the fresh faeces was suspended, contained K_2HPO_4 (8.8 g L^{-1}), KH_2PO_4 (6.8 g L^{-1}) (Merck, Darmstadt, Germany) and sodium thioglycolate (1 g L^{-1}) (Sigma-Aldrich, Steinheim, Germany).

Various liquid growth media were used throughout the different trials depending on the cultivated organisms (e.g. whole flora, specific bacterial isolates, etc.). For general preculturing and cultivation of porcine microorganisms, Brain Heart Infusion broth (BHI) (CM1135) (Oxoid Ltd., Hampshire, England) was used [28]. Specialized media were used for the cultivation of specific microbial strains: Sabouraud dextrose liquid medium (SDx) (CM0147) for fungi & yeasts, Wilkins-Chalgren anaerobe broth (WCh) (CM0643) for anaerobes, MacConkey broth (MacC) (CM0005) for coliforms and de Man Rogosa Sharpe broth (MRS) (CM0359) for lactic acid bacteria (Oxoid). Purified isolates were preserved as stocks containing Tryptic Soy broth (TSB) (CM131, Oxoid) and glycerol (1:3) (bidistilled (99.5%) (Analar Normapur VWR, Fontenay-sous-Bois, France)) at -80 °C. Supplementary, the microorganisms were stored at -80 °C in strain-specific media in case of activity-loss in general medium (e.g. MRS broth for lactic acid bacteria).

To improve anaerobic growth, L-cysteine (0.5 g L^{-1}) (SAFC Supply Solutions, St-Louis, USA) was added [32] to liquid media, while broths were pre-reduced by boiling, which further reduced the oxygen content.

For counting or isolation purposes agar media were made by adding bacteriological agar n°1 (LP0011, Oxoid) to the liquid broth medium of choice, which was heated until boiling and subsequently autoclaved.

To count bacteria present in fecal digestions an automated spiral plater was used (100 µL per plate) (EddyJet, IUL Instruments, Led Techno NV, Heusden-Zolder, Belgium). Plates were incubated in sealed jars containing AnaeroGen (3.5 L) (AN00035) sachets (Oxoïd) in a number according to the size of the jar to attain anaerobicity.

For the liquid digestion media, an N_2-flushing system equipped with sterile filters and needles was applied to obtain anaerobicity in the sterile digestion flasks. In order to

control its efficacy and to determine the required flushing time, dummy digestions were supplemented with resazurin (2 mg L^{-1}) (Sigma-Aldrich) and subsequently flushed causing extensive reduction of resazurin (blue) into resorufin (pink) and then hydroresorufin (colourless) over time, once anaerobic conditions were achieved [33].

2.3.2. Digestion protocol

The protocol applied for the static *in vitro* colonic digestion simulations in the present study was adapted from scientific literature on simulations of the gastrointestinal tract of humans and porcines [34,35,28].

2.3.2.1. Fecal inoculum and preculturing

Fecal slurry was attained by addition of 1/5 (S/L) phosphate buffered saline to the fresh faeces, followed by homogenisation in a stomacher for 10 minutes. The suspension was transferred into 50 mL falcon tubes and centrifuged at 500 × *g* for 2 minutes. To the supernatants 20% (v/v) of glycerol (99.5%) (Analar Normapur) was added as a cryoprotectant. This mixture was gently blended together obtaining a fecal inoculum, which was then stored at -80 °C.

Preculturing of the fecal inoculum before a 72 h *in vitro* colonic digestion was performed in BHI, unless mentioned otherwise (specialised media). Autoclaved penicillin flasks were filled with 36 mL of BHI broth and 4 mL of thawed fecal inoculum, flushed 1 h with N_2 (1 bar) and incubated (Innova 42, Incubator shaker series, New Brunswick Scientific) at 37 °C, 150 rpm for 24 h (= FI_{24}).

In a few digestion experiments thawed glycerol stocks from specific strains (-80 °C) were directly added to the rapeseed *in vitro* digestion (72 h), instead of first preculturing them (100 µL of stock was added to 40 mL of BHI).

2.3.2.2. Large intestinal simulation

Autoclaved penicillin flasks were supplemented with rapeseed (1.6 g) following a 1/25 (S/L) ratio. Subsequently, 40 mL of fluid was added consisting of a homogenous amount of FI_{24} precultered bacteria and BHI broth (1:1 ratio). The penicillin flasks were closed with vitton plugs allowing the establishment of anaerobic conditions after 1 h of flushing with N_2. The flasks were then incubated for 72 h at 37 °C at 150 rpm. Sampling occurred at the beginning and at end of the incubation (0 h and 72 h). In some set-ups samples were taken at other times specific to the experiment (24 h, 48 h or FI_{24}), or before any incubation ($FI_{-80°C}$).

2.3.3. Digestion set-ups

During a first digestion experiment, various porcine inocula (n=20) were incubated with rapeseed in order to detect a suitable high TU-producing fecal inoculum and to gain insight in the distribution of the occurrence of fecal TU formation. In a second experiment, the highest TU producing fecal inoculum was incubated both anaerobically and aerobically to evaluate the influence of oxygen on the *in vitro* digestion in BHI. In a third experiment, the influence of preculturing (24 h culturing before 72 h digestion), rapeseed addition, filter sterilization (0.22 µm, cellulose, Whatman™ syringe filters, Buckinghamshire, UK) and pasteurisation (60 °C for 0-10-30-60 min) of the precultured faecal inoculum FI_{24} was investigated. Filter sterilisation included a preliminary high-speed centrifugation step (20 min at 13,292 × *g*) to effectively remove fecal sludge prior to the use of the sterile filters, preventing leakage and clogging.

2.4. Bacterial identification

2.4.1. Isolation of thiouracil producing bacteria

The porcine inoculum with the highest TU production level was investigated for the presence of TU producing bacterial groups by using five different media upon *in vitro* rapeseed digestion: BHI as general medium (control), SDx for fungi & yeasts, WCh for anaerobes, MacC for coliforms and MRS for lactic acid bacteria (Oxoïd). As an exception 24 h preculturing of the faecal inoculum occurred in the respective specialised media instead of BHI, prior to the 72 h *in vitro* incubation with rapeseed (at 37 °C, 150 rpm). This porcine inoculum was also subjected to *in vitro* digestion with and without rapeseed addition. Next, both *in vitro* digestions were plated out in five different dilutions (1/10-1/100,000) on BHI and MacC agar plates (based on previous results: *3.3.1 Contributing microorganisms*) with a spiral plater and incubated under aerobic (24 h) and anaerobic conditions (for 48 h) at 37 °C. Based on colony morphology, different colonies from the rapeseed *in vitro* digestion were picked and re-evaluated for TU formation upon rapeseed digestion. The bacterial digests producing TU were subsequently plated out in four dilutions (1/10-1/10,000) under the starting conditions (aerobic or anaerobic and BHI or MacC). Similarly the morphologically divergent bacterial colonies were picked and evaluated through rapeseed *in vitro* digestions for TU formation. Also these digests were plated out under the starting conditions, but also in the opposed redox status (aerobic/anaerobic) to evaluate facultative aerobic or anaerobic traits. Hereupon the macroscopically different colonies were picked and concurrently incubated in rapeseed *in vitro*

digestions (TU detection) and subjected to streaking onto a general medium agar (BHI) to create monocultures. Once pure monocultures were achieved, the bacterial colonies were picked for a last *in vitro* evaluation of TU formation (with and without rapeseed).

2.4.2. Biochemical and genetic identification

Purified monocultures were phenotypically characterized by Gram-staining and API® 20E testing for preliminary identification (BioMérieux, Marcy-l'Etoile, France). Isolates showing an observed TU concentration increase of more than 60%, when rapeseed was added versus when no rapeseed added to the *in vitro* digestion (Table III.2), were selected for further identification tests.

For Gram-negative rods the oxidative-fermentative (OF) test developed by Hugh and Leifson [36] was applies, and of those capable to ferment glucose (indication for *Enterobacteriaceae*), a partial *rpoB* sequence was determined, as such sequences allow species discrimination within this family [37]. Total DNA was prepared according to the protocol of Niemann *et al*. [38]. The *rpoB* gene was amplified and sequenced using the primers described by Mollet *et al*. [37]. The PCR amplified *rpoB* gene product was purified using the NucleoFast 96 PCR Clean-up kit (Manchenery-Nagel, Düren, Germany). Sequencing reactions were performed using the BigDye® Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) and purified using the BigDye® XTerminator Purification Kit (Applied Biosystems). Sequencing was performed using an ABI Prism® 3130XL Genetic Analyzer (Applied Biosystems). Sequence assembly, the creation of a similarity matrix and the phylogenetic analysis were all accomplished with the use of the BioNumerics software 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity matrix was based on a pairwise alignment using an open gap penalty of 100% and a unit gap penalty of 0%. The phylogenetic analysis was performed after including the consensus sequence in an alignment of *rpoB* sequences collected from the international nucleotide sequence library EMBL and from LMG reference strains. A resulting tree was constructed using the neighbour-joining method.

Isolates identified through partial *rpoB*-sequencing, identification up to species level was not possible and therefore DNA-DNA hybridizations were performed. Template DNA for this method was isolated according to a modification of the procedure of Wilson [39]. Hybridizations were performed with DNA of a selected isolate and DNA of the type strain of *Escherichia coli* LMG 2092^T (BCCM/LMG Bacteria Collection, Ghent, Belgium) that was identified as the closest phylogenetic species of these isolates. Hybridizations were performed in the presence of 50% formamide at 43 °C according

to a modification [40,41] of the method described by Ezaki *et al*. [42]. Reciprocal reactions i.e. isolate DNA used as probe against DNA of LMG 2092^T (ATCC 25290) immobilized to a microplate well (A × B) and vice versa (B × A), were each performed in quadruplicate and the DNA-DNA relatedness was calculated from the mean value of A × B and that of B × A.

Isolates potentially belonging to the lactic acid bacteria (Gram-positive, oxidase- and catalase-negative) were subjected to AFLP™ DNA fingerprinting (Keygene N.V., Netherlands) for further identification. For this application DNA was prepared using the method of Gevers *et al.* [43] with slight modifications. AFLP™ DNA fingerprinting was performed as reported previously [44] using the restriction enzymes EcoR I and Taq I and the primer combination E01-6-FAM/T11 [45]. The obtained profiles were compared with reference profiles of lactic acid bacteria taxa (including *Bifidobacteria*) present in a BCCM/LMG in house database using the BioNumerics software. Clustering of the profiles was done using the Dice coefficient and the UPGMA algorithm.

2.5. Sample extraction and purification for thiouracil detection

Prior to the analysis of the digestion samples, sample preparation and clean up was needed. This procedure has been described [9]. Briefly, 5 mL of phosphate buffer (pH 8) was added to 1 mL of digest. The internal standard (PTU-d_5) was added at 50 µg kg^{-1} followed by 100 µL of a methanolic derivatisation solution containing 3-iodobenzyl bromide (2 mg mL^{-1}). Upon 10 minutes sonication, the derivatisation was allowed to proceed in the dark at 40 °C for 1 h. Afterwards the pH of the reaction mixture was adjusted to 3.6 (± 0.1) and different liquid-liquid extraction steps with 3, 2 and 2 mL of diethyl ether were applied. Once evaporated to dryness under a gentle N_2 stream (2 bar, 50 °C) the samples were dissolved in 100 µL CH_2Cl_2 and 300 µL cyclohexane. Further sample clean up consisted of solid phase extraction with silica cartridges conditioned with cyclohexane and eluted with a mixture of hexane/ethyl acetate 40/60 (v/v). Again N_2 evaporation was applied. Samples were redissolved in 160 µL of 0.5% acetic acid and methanol (50/50).

2.6. Standard addition

Quantification of TU in digestion samples was performed using the standard addition approach as described in Commission Decision 2002/657/EC [46]. Each sample was divided over 2 vials with analogous mass (m) and volume (V). One aliquot, the

unknown, was added with a mixture of 0.5% acetic acid and methanol 50/50 (V_A) and the other, the known, was enriched with an equal amount of analyte (TU) (V_{known}). The concentration of the addition solution was previously determined by analysing TU in digest samples and fitting these in a calibration curve in digestion buffer. This approximate estimation of the TU concentration was then spiked to the addition aliquot of each sample (ρ_A) giving rise to the area ratio of χ_{known}. Finally, quantification was established through evaluation of the area ratios of the known and unknown samples using the standard addition approach [21].

Quantification was established using the following formula: $C_{unknown} = \chi_{unknown} V_{unknown} \rho_A V_A / (\chi_{known} V_{known} m_{unknown} - \chi_{unknown} V_{unknown} m_{known})$, with $m_{unknown} = m_{known}$, $V_{unknown} = V_{known}$ and $C_{unknown} = \chi_{unknown} \rho_A V_A / (\chi_{known} - \chi_{unknown})$. In this formula, C, ρ are concentrations, χ is the area ratio, V is the volume, m is the mass and A is identified analyte (TU).

2.7. Instrumentation

2.7.1. *Liquid chromatography multiple mass spectrometry (LC-MS²)*

Detection of TU was achieved with a liquid chromatograph coupled to a linear ion trap mass spectrometer [21]. A Finnigan Surveyor LC-system (Thermo Electron, San Jose, USA) was combined with a Symmetry C18 column at 30 °C (5 µm x 150 mm x 2.1 mm, Waters, Milford, Massachusetts, USA) running on a 0.5% acetic acid (A) and methanol (B) 50/50 solvent combination at 0.3 mL min^{-1}. The linear gradient passed off as follows for 35 minutes: A/B 50/50 for 3 minutes, increasing to 0/100 in 17 minutes, and finally re-equilibrating for 10 minutes at 50/50. The linear ion trap mass spectrometer LTQ (Thermo Electron, San Jose, USA) was fitted with a heated electrospray ionization probe (HESI) operating in the negative ion mode. Applied working conditions were as follows: source voltage at 5 kV; capillary voltage at -50 V; tube lens voltage at -128.04 V; vaporizer and capillary temperature at 250 °C and 275 °C; sheath and auxiliary gas at 30 and 5 arbitrary units (a.u.), respectively. Based on its good in-house performance in urine and a secondary validation in digestion suspension, confirming these performance criteria (with a CC_α of 1.63 ng TU g^{-1} rapeseed, a mean recovery % of 102.73 ± 10.95 and a repeatability RSD% of 10.66), the method was found adequate for screening of TU in digests. Measured transitions are reported in Table III.1.

Table III.1: The monitored transitions by the LC-MS^2 for 2-thiouracil and its internal standard, 6-propyl-d_5-2-thiouracil.

Analyte	$[M-H]^-$	Product ions	Collision energy (eV)
2-thiouracil	343	182; 215; 309	44
6-propyl-d_5-2-thiouracil	390	127; 262; 356	30

2.8. Quality assurance

Preceding the LC-MS^2 analysis a standard mixture of the target compound and the internal standard were injected to check the operational conditions of the LC-MS^2 device. Identification of TU was based on the retention time relative to that of the internal standard, and the ion ratios of the product ions according to Commission Decision 2002/657/EC [46]. Every *in vitro* digestion simulation was conducted in three-fold and the internal standard was added prior to clean up (n=3).

2.9. Data handling

All data processing was performed with XCalibur 2.0.7 (Thermo Fisher Scientific, San Jose, CA, USA). Statistical testing (Student's-t-test) was carried out with SPSS 21 (IBM, Belgium) to assess significance ($p < 0.05$) of TU yields. Normality and equal variance were prerequisited.

3. RESULTS

3.1. Porcine intestinal thiouracil formation

TU detection resulting from 20 fecal porcine *in vitro* digestions with rapeseed ranged between 33.4 (± 0.1) and 80.5 (± 2.8) ng g^{-1} rapeseed for sows (S1-S10) and between 3.3 (± 0.8) and 16.5 (± 3.4) ng g^{-1} rapeseed for gilts (G1-G10) (Figure III.1). The gilts were female porcines, which were about to be inseminated for the first time, whereas the sows were multiparous adult females. Statistical difference was proven ($p < 0.001$) between both life stages (sow-gilt) regarding their TU production upon rapeseed digestion.

Furthermore, anaerobic and aerobic conditions were compared for the rapeseed-based TU production. Under anaerobic conditions, TU production was significantly (p

= 0.015) higher compared to the aerobic digestion at the end of the incubation (T_{48}), respectively, 55.04 ± 4.73 ng g^{-1} rapeseed and 17.70 ± 8.86 ng g^{-1} rapeseed.

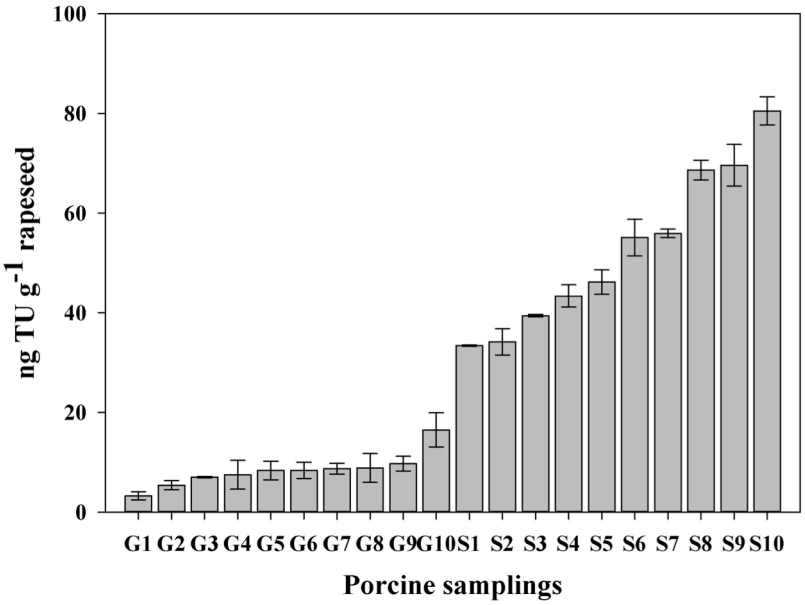

Figure III.1: Results of thiouracil (TU) production in 10 gilts (G1-G10) and 10 sows (S1-10) during *in vitro* digestive simulations with rapeseed (n=3) for 72 hours (ng g^{-1} rapeseed (mean ± SD)).

3.2. *In vitro* colon digestion & thiouracil formation: influential factors

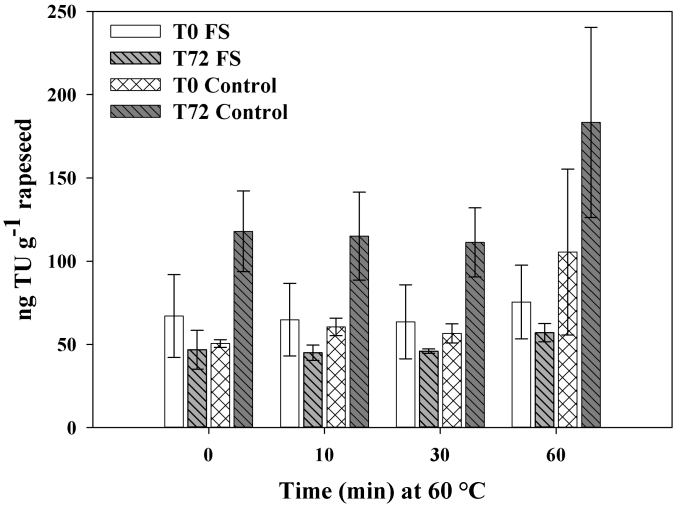

Figure III.2: Effect of filter sterilisation (FS) and pasteurisation (0-10-30-60 min at 60°C) of the pre-cultured fecal porcine inoculum (FI_{24}) on thiouracil formation of a high-producing porcine fecal inoculum during *in vitro* digestive simulation with rapeseed (ng TU g^{-1} rapeseed (mean ± SD)) (n=3) at the start (T0) and towards the end of the incubation (T72).

To explore the mechanism behind TU formation, a high TU producing porcine inoculum was exposed to either various periods (0-10-30-60 minutes) of pasteurization (60 °C) or filter sterilization (0.22 µm) or a combination of both prior to a rapeseed *in vitro* digestive simulation. No significant increase in TU formation could be demonstrated after 72h of digestion upon filter sterilisation in any combination with pasteurisation (Figure III.2). Pasteurisation alone did not affect the TU forming potential of the fecal inoculum since a significant ($p = 0.048$) TU increase similar to that of the control ($p = 0.036$) could be seen up to 30 min into pasteurisation (60 °C). Beyond this duration, variation in TU formation increased, but no significant difference in TU formation was noticed (Figure III.2).

To identify the origin of the background TU levels prior to digestive simulation, *in vitro* incubations of BHI broth as such, non a priori cultivated fecal inoculum (FI_{-80} °C), FI_{24}, rapeseed (RS) and autoclaved rapeseed (autocl. RS) in specific combinations, were performed (Figure III.3). Trace levels of TU in thawed fecal inoculum ($T_{-80°C}$) prior to any incubation and in incubated (T_{72}) BHI medium were observed. After 24h (FI_{24}) of pre-culturing the fecal inoculum (FI), a significant ($p < 0.05$) increase in TU concentration could be detected as compared to T_0.

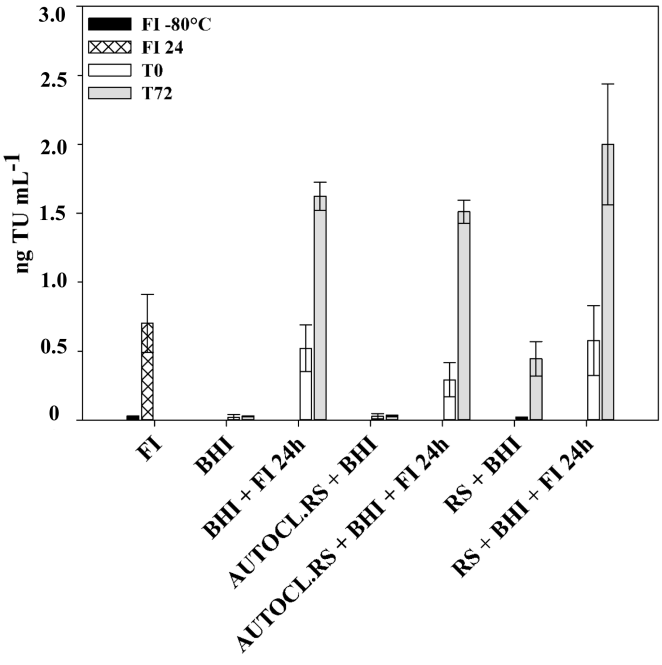

Figure III.3: Overview of the effects on thiouracil formation of rapeseed (RS), autoclaved rapeseed (autocl. RS), and brain heart infusion by a whole porcine fecal inoculum at the start (T0) and towards the end of the incubation (T72). In addition, thawed fecal inoculum ($FI_{-80°C}$) and precultured fecal inoculum (FI_{24}) were evaluated for thiouracil formation (ng TU mL^{-1} (mean ± SD)) (n=3).

After 72h of incubation the *in vitro* digestion of Fl_{24} and BHI without rapeseed produced similar amounts of TU compared to the same digestion respectively, with rapeseed and with autoclaved rapeseed. The digestions that did not contain Fl_{24} showed no significant increase of TU over time, except for BHI with rapeseed, which showed a significant ($p = 0.027$) but minor increase of TU compared to T_0.

3.3. Bacterial isolation and identification

3.3.1. Contributing microorganisms

To preliminary identify bacterial groups, potentially contributing to TU formation upon *Brassicaceae* digestion, the porcine fecal inoculum with the highest TU production level was incubated in one non-selective BHI and four group-specific broths. TU production was the highest in MacC (39.5 ± 1.9% TU) and MRS (25.8 ± 2.8% TU) media relative to the non-selective BHI (100% TU) (Figure III.4).

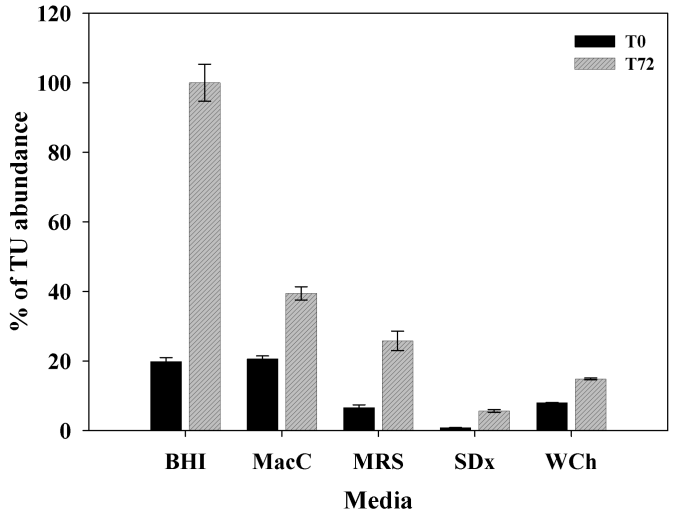

Figure III.4: Overview of thiouracil production (%) by a high thiouracil producing porcine fecal inoculum in different broths (MacC: coliforms; MRS: lactic acid bacteria; SDx: anaerobes; WCh: fungi and yeasts) after pre-culturing prior to incubation (T_0) and after 72 hours of incubation with rapeseed (T_{72}) (% of TU abundance (mean ± SD)) (n=3).

To identify and isolate TU producing bacterial species, the highest TU producing intestinal culture inoculum of a sow was selected for *in vitro* digestion, with and without rapeseed addition. Finally, thirteen fecal suspensions were found positive for TU after this process, from which only 8 digestions were selected with sufficient TU production ranging from 18.47 to 31.88 ng g^{-1} rapeseed.

Table III.2: Identification of thiouracil producing bacteria: thiouracil-% (TU%) increase between rapeseed (RS)-control digestions, morphological, biochemical and genetic identification (n.d: not defined; no ID: no matching identification from database).

Identified bacterial species	TU% increase (with vs. without RS)	Cell morphology	Gram stain	Oxidase	Catalase	Fermentative	Identification procedures	
							API® 20E	Genetic identification
E. coli	88.1	rods (0.7x1.0-1.8 µm), single, pairs, nonmotile	-	-	+	+	no ID	*rpoB* sequence analysis, DNA-DNA hybridization (ATCC 25290): *Escherichia coli*
E. coli	31.8	rods	-	n.d.	n.d.	n.d.	*E. coli 1 (98.8%)*	n.d.
E. coli	90.6	rods (0.9x1.5-5.0 µm), single, pairs, nonmotile	-	-	+	+	*E. coli 1 (88.3%)*	*rpoB* sequence analysis, DNA-DNA hybridization (ATCC 25290): *Escherichia coli*
E. coli	26.5	rods	-	n.d.	n.d.	n.d.	*E. coli 1 (88.3%)*	n.d.
Salmonella enterica ssp. arizonae	62.4	rods	-	n.d.	n.d.	n.d.	*Salmonella enterica ssp. arizonae (99.1%)*	n.d.
E. coli	89.6	rods (0.9x1.5-10 µm), single, pairs, nonmotile	-	-	+	+	*Enterobacter cloacae (38.8 %)*	*rpoB* sequence analysis, DNA-DNA hybridization (ATCC 25290): *Escherichia coli*
L. reuteri	93.7	rods (1.0x1.5-3.0 µm), single, pairs, nonmotile	+	-	-	/	no ID	AFLP™: *Lactobacillus reuteri*
L. reuteri	89.4	rods (1.0x1.5-3.0 µm), single, pairs, nonmotile	+	-	-	/	no ID	AFLP™: *Lactobacillus reuteri*
E. faecium	82.87	cocci Ø 0.9 µm, single, pairs, short chaines, nonmotile	+	-	-	/	*Pantoea spp. 1 (48.9%)*	AFLP™: *Enterococcus faecium*

Next, 12 pure monocultures were achieved and investigated using API® 20E. Only four of these isolates could be identified directly based on the APIweb™ database: three as *Escherichia coli* (ID%: 98.0% - 88.3% - 88.3%) and one as *Salmonella enterica ssp. arizonae* (ID%: 99.1%). From the remaining isolates, 3 were not further enquired, because they were not producing high amounts of TU. The remaining five isolates showed an observed TU concentration increase of more than 80% when rapeseed was added to the *in vitro* digestion in comparison to when none was added (Table

III.2). These isolates and a previously by API identified *E. coli* representative (90.6 TU% increase) were selected for further identification.

Three isolates were identified as *Escherichia* spp., probably *E. coli* based on *rpoB* gene sequence analyses, while three others were identified as lactic acid bacteria (one as *Enterococcus faecium* and two as *Lactobacillus reuteri*) based on AFLP™ DNA fingerprinting. One representative *Escherichia* isolate was additionally investigated through DNA-DNA hybridization. DNA of this isolate was compared with DNA of the type strain of *E. coli* LMG 2092^T) and 75% DNA-DNA relatedness was found, which is above 70%, the value generally regarded as the limit of species delineation (47). Therefore, the *Escherichia* isolates were confirmed to belong to *E. coli.* In total, nine isolates were identified at the species level through the different identification procedures (Table III.2). Their respective TU increase upon rapeseed *in vitro* incubation is presented in Figure III.5.

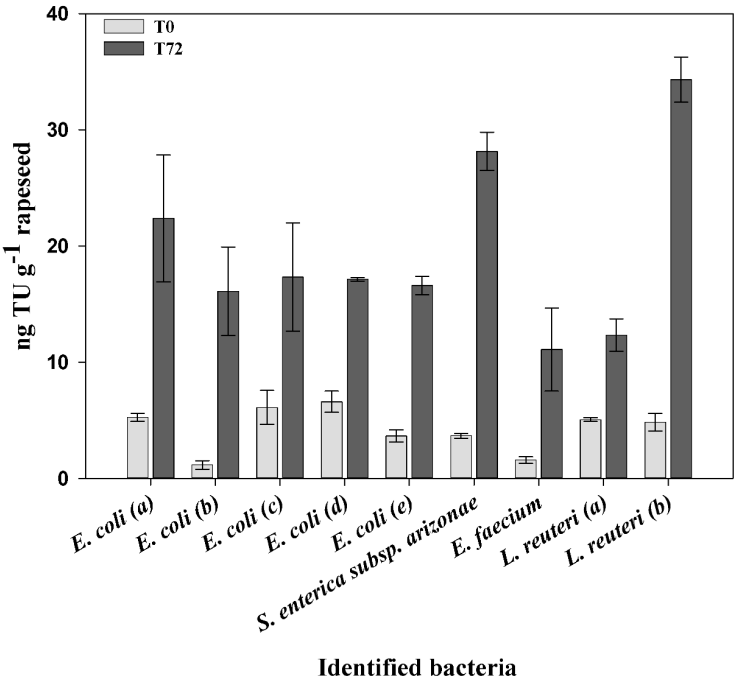

Figure III.5: Recovered thiouracil concentrations (ng g^{-1} rapeseed (mean ± SD)) during pre-cultured *in vitro* incubations with rapeseed (n=3) of the 9 identified TU producing isolates (*Enterococcus faecium, Escherichia coli (a-e), Lactobacillus reuteri (a-b), Salmonella enterica ssp. arizonae*) at the start (T_0) and towards the end of the incubation (T_{72}).

To elucidate precursors in the substrate of microbial TU formation, *Lactobacillus reuteri* and *Enterococcus faecium*, as high and low TU producing representatives (Figure III.5) were exposed to various (rapeseed, autoclaved rapeseed and no rapeseed) incubations. For both bacteria a similar pattern was observed with significantly ($p < 0.05$) higher TU concentrations when rapeseed was added as compared to autoclaved rapeseed and control (no rapeseed) incubations (Figure III.6). TU concentrations in autoclaved rapeseed incubations were not significantly ($p > 0.05$) different from the controls.

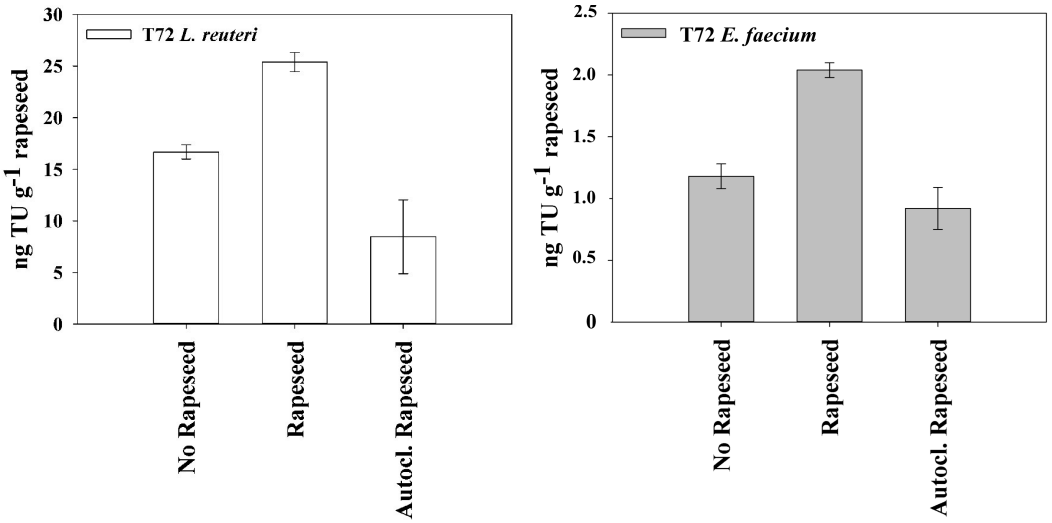

Figure III.6: Influence of rapeseed on thiouracil production (ng g^{-1} (mean ± SD)) upon *in vitro* incubation (n=3) with purified *Lactobacillus reuteri* and *Enterococcus faecium* (not precultured).

Thereupon, *Lactobacillus reuteri, Enterococcus faecium* and also *Escherichia coli* were plated onto BHI agar and onto the more selective agars: MRS (*Lactobacillus reuteri, Enterococcus faecium)* and MAcC (*Escherichia coli),* for enumeration after incubation with and without rapeseed, without preculturing the organisms to evaluate whether the bacteria developed sufficiently over time and rapeseed influenced bacterial growth. Results showed the $\log_{10}$ ranged between 7 - 9 mL^{-1} at T_{72}. No significant difference was found between rapeseed and control incubations regarding bacterial counts, except for *L. reuteri*, which had a positive significant ($p < 0.05$) increase in bacterial count in the rapeseed digestion (8.23 ± 0.35 $\log_{10}$ mL^{-1}) compared to the control (7.11 ± 0.35 $\log_{10}$ mL^{-1}) digestion.

4. DISCUSSION

In the present study, 9 cultures belonging to four different bacterial species, *E. coli, E. faecium, L. reuteri and Salmonella enterica ssp. arizonae* were isolated from porcine feces and identified as involved in the production of the banned thyreostat thiouracil upon rapeseed incubation. The mechanism bringing about endogenous thiouracil was further enquired by altering digestion parameters, providing strong evidence that an extracellular membrane-bound bacterial enzyme (myrosinase-like) formed under anaerobic conditions and the addition of rapeseed were pivotal in this process.

The *in vitro* microbial intestinal TU formation observed in this study, was significantly different between porcine life stages. It has indeed been reported that the diversity of the intestinal microbiota in pigs increases with age [48,49,50], which could explain the higher TU producing fecal inocula derived from elder sows. Moreover, the presence of *E. coli* and *Lactobacillus sp.* [48,50] has been described as lower in young pigs. For *E. coli* specifically, Katouli *et al*. [51] showed the mean number of biochemical phenotypes in piglets increased as animals aged and sows normally had a higher number of biochemical phenotypes than their offspring. They subsequently reported that the dominant *E. coli* strains in piglets are quite different from those of their sows. Therefore, the number of different intestinal bacterial strains might potentially be important to intestinal TU formation, as well as, the present dominant types of a strain [51]. These findings suggest that colonic bacterial fermentation significantly contributes to the prevalence of endogenous thiouracil in livestock urine. Of course, relating these findings to *in vivo* data remains difficult. It is however likely that naturally formed thiouracil residues are excreted in urine upon absorption through the intestinal tract, which has been previously observed *in vivo* with below 10 ppb TU concentrations upon Brassicaceae feeding in bovines [1]. Orally administered thyreostatic drugs have been reported to rapidly occur in urine and reach a cumulative total of 6.49-16.7% of the administered dose within 24h in rats [52]. Whether this excretion rate based on oral administration is also applicable to the excretion of intestinally formed TU remains to be investigated.

Notwithstanding, the detection of TU in urine samples of livestock is a widespread phenomenon, but urinary residues are not consequently recorded in every animal. A survey study from national control plan data in France recalled 32% of TU positive samples in bovines (n=1089) and 80% in porcines (n=201) [53]. This study showed that concentrations of TU were higher in bovines than in porcines considering only porcines between 1 and 8 months old, which from our results show significantly lower

TU producing potential *in vitro*. In a Polish survey only 14% bovines (n=279) and 13% pigs (n=258) tested positive for TU, but no age categories were mentioned here [54]. In this context, the influence of age, gender and in a minor way country on the determination of a potential TU threshold value have been described *in vivo* [53,54].

To propose a mechanistical explanation for TU formation upon Brassicaceae digestion, a high producing porcine intestinal inoculum was evaluated under variable *in vitro* conditions. The use of various selective media pointed towards the involvement of coliform and lactic acid bacteria, which are abundantly present in the gut microbial communities of pigs [55,29]. Moreover, further elucidation of the influence of redox conditions sustained the fact that anaerobic microorganisms outnumber aerobic bacteria by at least a factor 3 to 5 $\log_{10}$ [49] in the intestinal environment of pigs. This explains the lower TU concentration monitored under aerobic conditions. Filter sterilisation showed no increase of TU concentration upon *in vitro* digestion, providing evidence for the requirement of (active) bacterial cells for TU formation to take place. Background TU traces present prior to incubation may be attributed to the pre-culturing of bacteria in the presence of other fecal constituents. During this period, the fecal inoculum can metabolize any feed-related remains, which are inherently present in the fecal suspension, consequently resulting in the production of TU. Indeed, when no pre-culturing was applied, TU formation was marginal. Pasteurisation of the pre-cultured inoculum did not significantly affect TU formation. It has been described that *E. coli* can survive at 60 °C up to 30 minutes depending on strain and surrounding matrix [56]. At 55 °C, inactivation is only achieved after 60-120 minutes in broth [56]. Although heat-treated microorganisms generally do not grow on agar, they can succeed in repairing damage and regain their potential for growth under optimal broth conditions [56]. Such events might explain the undisturbed TU production over the first 30 minutes of pasteurisation, while after 60 min bacterial survival is unlikely due to irreversible damage. In the assumption that the responsible (bacterial) mediator is a membrane-bound protein (myrosinase-like enzyme), heat treatment would likely cause some degree of denaturation over time, but semi-reversible denaturation has been reported [57]. Moreover, β-thioglucoside glucohydrolases remain active in a broad range of temperatures (up to 60 °C) and pHs (5-10) [58], offering an explanation for sustained TU production upon pasteurization and supporting the potential hypothesis of an extracellular membrane-bound myrosinase-like enzyme as mediating factor for TU formation.

The identified bacterial isolates with a TU forming ability include some of the most prevalent intestinal bacteria, i.e. lactobacilli, coliforms and enterococci populating the intestine of pigs [49,59]. Since these bacteria are common residents of the intestine, in many animal species, the occurrence of TU in urine of several animal species as reported earlier, may be easily explained [2]. *Enterococcus faecium* is known as the most prevalent *Enterococcus* sp. in porcines [60] and has also been described to metabolize glucosinolates *in vitro* [61]. Both *Lactobacillus* sp. and *Enterococcus* sp. have been demonstrated to express ß-thioglucosidase activity [62]. In a study with rats transfaunated with *Lactobacillus sp.* (LEM 220) and fed with rapeseed meal, an increased goitrogenicity was observed as compared to germ-free rats [26,63]. Similar results were obtained in gnotobiotic *E. coli* (EM0) mice, when fed rapeseed meal [64]. Moreover, degradation of progoitrine, a precursor of goitrine (natural thyeostat), by *E. coli* [65] and the glucosinolate sinigrin by *L. agilis (R16)* [27] have also been described. Specifically in rodents, pigs and chickens, *L. reuteri* is one of the dominant species in the gastrointestinal tract [66]. This lactic acid bacterium has the capacity to form reuterin, an antimicrobial agent, and has therefore been proposed as a potential additive for pelleted feeds in pigs [67]. Two of the prerequisited characteristics for pelleting was resistance to 70 °C for 10 seconds and to gastric and intestinal pHs. *Previously known as S. choleraesuis ser. arizonae*, the later renamed *S. enterica ssp. arizonae*, has been isolated from reptiles, fowl, turkeys, ducks, dogs, cats, monkeys, goats, wild boars and lately from pigs [68], but no glucosinolate degrading or myrosinase-like capacities have been described yet for this taxon.

To confirm Brassicaceae feed as the source for TU formation, *L. reuteri* and *E. faecium* as high and low TU producing bacteria, respectively, and a high TU producing porcine fecal inoculum were selected for incubations. Autoclaved rapeseed and control incubations with pure strains did not differ significantly in TU concentrations. Addition of rapeseed as a whole did however significantly increase TU formation upon incubation with pure strains, reassuring its importance as a source of TU precursors in line with previous *in vitro* [28] and *in vivo* [1] reports. During *in vitro* digestion with the high producing porcine fecal inoculum, TU formation did not significantly differ between autoclaved and non-autoclaved rapeseed administration. This may be explained by the prior assumption that fecal constituents present in the inoculum can act as precursors for TU formation when no rapeseed is added. Indeed, in literature it has been described that 60% of the glucosinolate content of feed reaches the colon unaltered in pigs and although these are mostly degraded prior to

defecation, many breakdown products are present in feces [69]. The autoclaved rapeseed and control *in vitro* digestions produced equal amounts of TU upon incubation with fecal inoculum and pure strains. Autoclaved, and thus sterile and possibly denaturated rapeseed may not contain the required precursors and as a result not differ significantly from absence of rapeseed. Oerlemans *et al.* [70] reported that at temperatures above 110 °C substantial breakdown of all glucosinolate groups was observed, which may also be the case during autoclavation (121 °C). Besides, it is possible that the rich BHI medium, which consists out of animal tissues (brain, heart), is additionally responsible for low TU levels in bacterial incubations without rapeseed or autoclaved rapeseed, since it has been reported that 2-thiouracil can be found in tRNA of *E. coli* and that *N*-glucosidases from *E. coli* extracts are able to cleave uracil free from DNA, which is one of its building blocks [71].

Finally, the influence of the substrate (rapeseed) was evaluated on bacterial growth through enumeration of some of the pure strains, to assure that increased bacterial growth was responsible for enhanced TU formation and not feed itself. Indeed, it has been described that certain hydrolysis products from various *Brassica* crops exert *in vitro* inhibiting influences on bacterial growth of *Enterobacteriaceae* and certain *Enterococci* in pigs [72]. In this study however, no negative effects on bacterial growth were observed as a result of rapeseed administration for *E. coli* and *E. faecium*. In contrast, for *L. reuteri* minor growth stimulation was observed when rapeseed was added.

The absence of effect of rapeseed on bacterial growth and its restricted growth stimulation in case of *L. reuteri* may be explained by the variety of glucosinolates present in rapeseed, which are mainly of aliphatic origin. In the study by Saavedra *et al.* [72] the effect of glucosinolate hydrolysis products on intestinal bacteria in pigs was investigated. Mainly indolic and aromatic glucosinolates, but also two aliphatics (sinigrin and glucoraphanin) were considered. Depending on the type of hydrolysis product, its concentration, as well as, the type of bacteria, which was exposed to it, the inhibiting effect can be hampered. From these results, it may be concluded that TU formation is directly related to the presence of rapeseed feed and its inherent glucosinolates and/or degradation products thereof, but other sources of TU precursors may not be excluded.

In summary, fecal inocula of porcines in two different physiological stages of their life were screened for their capability to produce the banned thyreostat thiouracil upon rapeseed digestion, resulting in the observation of an age-increasing microbial capacity to produce TU. Furthermore, 9 TU forming bacteria were isolated from a

high-producing sow and identified as belonging to the species *Escherichia coli, Enterococcus faecium, Lactobacillus reuteri* and *Salmonella enterica ssp. arizonae*. Moreover, the mechanism behind this bacteria-mediated formation was tentatively characterised, demonstrating that a cell-dependent, membrane-bound, moderate heat resistant (60 °C up to 30 min) enzyme was involved. In addition, it may be concluded that rapeseed as a feed can be considered as a possible source of TU precursors, sustaining the hypothesis that glucosinolates subjected to a bacterial myrosinase-like enzyme activity from the basis of this observation. These new insights in endogenous TU formation in livestock, offer a mechanistical explanation for its low-level observation, but foremost reinforce the need for a more tailored EU legislation.

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6. REFERENCES

[1] **Pinel, G., S. Mathieu, N. Cesbron, D. Maume, H. F. De Brabander, F. Andre, and B. Le Bizec.** 2006. Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyrostats in meat production. Food Additives and Contaminants **23:**974-980.

[2] **Vanden Bussche, J., L. Vanhaecke, Y. Deceuninck, K. Wille, K. Bekaert, B. Le Bizec, and H. F. De Brabander.** 2011. Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry detection of naturally occuring thiouracil in urine of untreated livestock, domesticated animals and humans. Food Additives and Contaminants **28:**166-172.

[3] **European Community.** 1981. Council Directive 81/602/EC. Official Journal of the European Communities **L 222:**32-33.

[4] **International Agency for Research on Cancer.** 2011. IARC monographs on the evaluation of carcinogenic risks to humans.
Available from: http://monographs.iarc.fr/ENG/Classification/index.php.

[5] **Khera, K. S.** 1973. Ethylenethiourea - Teratogenicity Study in Rats and Rabbits. Teratology **7:**243-252.

[6] **Martinez-Frias, M. L., A. Cereijo, E. Rodriguezpinilla, and M. Urioste.** 1992. Methimazole in Animal Feed and Congenital Aplasia Cutis. Lancet **339:**742-743.

[7] **Ebrahimzadeh, H., A. A. Asgharinezhad, L. Adlnasab, and N. Shekari.** 2012. Optimization of ion-pair based hollow fiber liquid phase microextraction combined with HPLC-UV for the determination of methimazole in biological samples and animal feed. Journal of Separation Science **35:**2040-2047.

[8] **European Community.** 1996. Council Directive 96/23/EC. Official Journal of the European Communities **L 125:**10-32

[9] **Pinel, G., E. Bichon, K. Pouponneau, D. Maume, F. Andre, and B. Le Bizec.** 2005. Multi-residue method for the determination of thyreostats in urine samples using liquid chromatography coupled to tandem mass spectrometry after derivatisation with 3-iodobenzylbromide. Journal of Chromatography A **1085:**247-252.

[10] **Lõhmus, M., K. Kallaste, and B. Le Bizec.** 2009. Determination of thyreostats in urine and thyroid gland by ultra high performance liquid chromatography tandem mass spectrometry. Journal of Chromatography A **1216:**8080-8089.

[11] **Vanden Bussche, J., L. Vanhaecke, Y. Deceuninck, K. Verheyden, K. Wille, K. Bekaert, B. Le Bizec, and H. F. De Brabander.** 2010. Development and validation of an ultra-high performance liquid chromatography tandem mass spectrometry method for quantifying thyreostats in urine without derivatisation. Journal of Chromatography A **1217:**4285-4293.

[12] **Pinel, G., D. Maume, Y. Deceuninck, F. Andre, and B. Le Bizec.** 2006. Unambiguous identification of thiouracil residue in urine collected in non-treated bovine by tandem and high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry **20:**3183-3187.

[13] **European Food Safety Authority.** 2010. Report for 2008 on the results from the monitoring of veterinary medicinal product residues and other substances in food of animal origin in the Member States. European Food Safety Authority Journal **8**:1559. doi:10.2903/j.efsa.2010.1559.

[14] **European Union Reference Laboratories,** 2007. EURL guidance document. Available online on: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf

[15] **Zukalová, H., and J. Vašák .** 2002. The role and effects of glucosinolates of Brassica species - a review. Rostlinna Vyroba **48:**175-180.

[16] **Bones, A. M., and J. T. Rossiter.** 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. Physiologia Plantarum **97:**194-208.

[17] **Rosa, E. A. S., and A. S. Rodrigues.** 2001. Total and individual glucosinolate content in 11 broccoli cultivars grown in early and late seasons. Hortscience **36:**56-59.

[18] **Wallig, M. A., R. L. Belyea, and M. E. Tumbleson.** 2002. Effect of pelleting on glucosinolate content of Crambe meal. Animal Feed Science and Technology **99:**205-214.

[19] **Ahlin, K. A., M. Emanuelson, and H. Wiktorsson.** 1994. Rapeseed Products from Double-Low Cultivars as Feed for Dairy-Cows - Effects of Long-Term Feeding on Thyroid-Function, Fertility and Animal Health. Acta Veterinaria Scandinavica **35:**37-53.

[20] **European Community.** 2003. Commission Regulation No. 1035/2003/EC. Official Journal of the European Communities **L 150**:351-357.

[21] **Vanden Bussche, J., J. A. L. Kiebooms, N. De Clercq, Y. Deceuninck, B. Le Bizec, H. F. De Brabander, and L. Vanhaecke.** 2011. Feed or Food Responsible for the Presence of Low-Level Thiouracil in Urine of Livestock and Humans? Journal of Agricultural and Food Chemistry **59:**5786-5792.

[22] **Krul, C., C. Humblot, C. Philippe, M. Vermeulen, M. van Nuenen, R. Havenaar, and S. Rabot.** 2002. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic *in vitro* large intestinal model. Carcinogenesis **23:**1009-1016.

[23] **Cheng, D. L., K. Hashimoto, and Y. Uda.** 2004. *In vitro* digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. Food and Chemical Toxicology **42:**351-357.

[24] **Tripathi, M. K., and A. S. Mishra.** 2007. Glucosinolates in animal nutrition: A review. Animal Feed Science and Technology **132:**1-27.

[25] **Elfoul, L., S. Rabot, N. Khelifa, A. Quinsac, A. Duguay, and A. Rimbault.** 2001. Formation of allyl

isothiocyanate from sinigrin in the digestive tract of rats monoassociated with a human colonic strain of *Bacteroides thetaiotaomicron*. Fems Microbiology Letters **197:**99-103.
[26] **Nugonbaudon, L., S. Rabot, J. M. Wal, and O. Szylit.** 1990. Interactions of the intestinal microflora with glucosinolates in rapeseed meal toxicity - 1st Evidence of an intestinal *Lactobacillus* possessing a myrosinase-like activity *In vivo*. Journal of the Science of Food and Agriculture **52:**547-559.
[27] **Palop, M. L., J. P. Smiths, and B. Tenbrink.** 1995. Degradation of sinigrin by *Lactobacillus agilis* strain R16. International Journal of Food Microbiology **26:**219-229.
[28] **Kiebooms, J. A. L., J. Vanden Bussche, L. Y. Hemeryck, V. Fievez, and L. Vanhaecke.** 2012. Intestinal Microbiota Contribute to the Endogenous Formation of Thiouracil in Livestock. Journal of Agricultural and Food Chemistry **60:**7769-7776.
[29] **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Applied and Environmental Microbiology **68:**673-690.
[30] **Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon.** 2008. Evolution of mammals and their gut microbes. Science **320:**1647-1651.
[31] **Rozeboom, D. W., J. E. Pettigrew, R. L. Moser, S. G. Cornelius, and S. M. ElKandelgy.** 1996. Influence of gilt age and body composition at first breeding on sow reproductive performance and longevity. Journal of Animal Science **74:**138-150.
[32] **Shanson, D. C., and J. Singh.** 1981. Effect of adding cysteine to brain-heart infusion broth on the isolation of *Bacteroides fragilis* from experimental blood cultures. Journal of Clinical Pathology **34:**221-223.
[33] **O'Brien, J., I. Wilson, T. Orton, and F. Pognan.** 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry **267:**5421-5426.
[34] **Boisen, S. and J. A. Fernandez.** 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. Animal Feed Science and Technology **68:**277-286.
[35] **Gawlik-Dziki, U., D. Dziki, B. Baraniak, and R. F. Lin.** 2009. The effect of simulated digestion *in vitro* on bioactivity of wheat bread with Tartary buckwheat flavones addition. Lwt-Food Science and Technology **42:**137-143.
[36] **Hugh, R., E. Leifson.** 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative rods. Journal of Bacteriology **66**:24–26.
[37] **Mollet, C., M. Drancourt, and D. Raoult.** 1997. *rpoB* sequence analysis as a novel basis for bacterial identification. Molecular Microbiology **26:**1005-1011.
[38] **Niemann, S., A. Puhler, H. V. Tichy, R. Simon, and W. Selbitschka.** 1997. Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural Rhizobium meliloti population. Journal of Applied Microbiology **82:**477-484.
[39] **Wilson, K.** 1978. Preparation of genomic DNA from bacteria. In: Current Protocols in Molecular Biology, pp. 2.4.1-2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl. Green Publishing and Wiley-Interscience, New York.
[40] **Goris, J., K. Suzuki, P. De Vos, T. Nakase, and K. Kersters.** 1998. Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. Canadian Journal of Microbiology **44:**1148-1153.
[41] **Cleenwerck, I., K. Vandemeulebroecke, D. Janssens, and J. Swings.** 2002. Re-examination of the genus *Acetobacter,* with descriptions of *Acetobacter cerevisiae sp nov* and *Acetobacter malorum sp nov*. International Journal of Systematic and Evolutionary Microbiology **52:**1551-1558.
[42] **Ezaki, T., Y. Hashimoto, and E. Yabuuchi.** 1989. Fluorometric Deoxyribonucleic Acid Deoxyribonucleic Acid Hybridization in Microdilution Wells as an Alternative to Membrane-Filter Hybridization in Which Radioisotopes Are Used to Determine Genetic Relatedness among Bacterial Strains. International Journal of Systematic Bacteriology **39:**224-229.
[43] **Gevers, D., G. Huys, and J. Swings.** 2001. Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. Fems Microbiology Letters **205:**31-36.
[44] **Chimetto, L. A., I. Cleenwerck, M. Brocchi, A. Willems, P. De Vos, F. L. Thompson.** 2011. *Marinomonas brasilensis* sp. nov., isolated from the coral *Mussismilia hispida* ,and reclassification of *Marinomonas basaltis* as a later heterotypic synonym of *Marinomonas communis*. International Journal of Systematic and Evolutionary Microbiology **61:**1170-1175.
[45] **Sistek, V., A. F. Maheux, M. Boissinot, K. A. Bernard, P. Cantin, I. Cleenwerck, P. De Vos, M. G. Bergeron.** 2012. *Enterococcus ureasiticus* sp. nov. and *Enterococcus quebecensis* sp. nov., isolated from water. Journal of Systematic and Evolutionary Microbiology **62:**1314-1320.
[46] **European Community**. 2002. Council Decision 2002/657/EC. Official Journal of the European Communities **L 221**:8-36.
[47] **Wayne, L. G., D. J. Brenner, R. R. Colwel, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray & other authors**. 1978. International Committee on Systemic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Journal of Systematic and Evolutionary Microbiology **37:**453-464.
[48] **Wilbur, R. D., D. V. Catron, L. Y. Quinn, V. C. Speer, and V. W. Hays.** 1960. Intestinal Flora of the Pig as Influenced by Diet and Age. J Nutr **71:**168-175.
[49] **Katouli, M. and P. Wallgren.** 2005. Chapter 2: Metabolism and population dynamics of the intestinal microflora in growing pigs in Microbial ecology in growing animals. W. H. Holzapfel, P. J.

Naughton (eds.), Elsevier Ltd., Philadelphia, pp. 21-53. ISBN: 978-0-444-50926-0.
[50] **Kim, H. B., K. Borewicz, B. A. White, R. S. Singer, S. Sreevatsan, Z. J. Tu, and R. E. Isaacson.** 2011. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. Veterinary Microbiology **153:**124-133.
[51] **Katouli, M., A. Lund, P. Wallgren, I. Kuhn, O. Soderlind, and R. Mollby.** 1995. Phenotypic Characterization of Intestinal Escherichia-Coli of Pigs during Suckling, Postweaning, and Fattening Periods. Applied and Environmental Microbiology **61:**778-783.
[52] **Pittman, J. A., R. J. Beschi, and Smitherm.Tc.** 1971. Methimazole - Its Absorption and Excretion in Man and Tissue Distribution in Rats. Journal of Clinical Endocrinology & Metabolism **33:**182.
[53] **Le Bizec, B., E. Bichon, Y. Deceuninck, S. Prevost, F. Monteau, J. P. Antignac, and G. Dervilly-Pinel.** 2011. Toward a criterion for suspect thiouracil administration in animal husbandry. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment **28:**840-847.
[54] **Wozniak, B., S. Witek, J. Zmudzki, and A. Klopot.** 2012. Natural Occurrence of Thiouracil in Urine of Livestock in Poland. Bulletin of the Veterinary Institute in Pulawy **56:**611-615.
[55] **Robinson, I. M., S. C. Whipp, J. A. Bucklin, and M. J. Allison.** 1984. Characterization of Predominant Bacteria from the Colons of Normal and Dysenteric Pigs. Applied and Environmental Microbiology **48:**964-969.
[56] **Usajewicz, I. and B. Nalepa.** 2006. Survival of Escherichia coli O157 : H7 in milk exposed to high temperatures and high pressure. Food Technology and Biotechnology **44:**33-39.
[57] **Dewit, J. N. and G. Klarenbeek.** 1984. Effects of Various Heat-Treatments on Structure and Solubility of Whey Proteins. Journal of Dairy Science **67:**2701-2710.
[58] **Zhou, C. H., J. G. Tokuhisa, D. R. Bevan, and A. Esen.** 2012. Properties of beta-thioglucoside hydrolases (TGG1 and TGG2) from leaves of Arabidopsis thaliana. Plant Science **191:**82-92.
[59] **Allison, M. J.** 1989. Characterization of the flora of the large bowel of pigs-a status-Report. Animal Feed Science and Technology **23:**79-90.
[60] **Devriese, L. A., J. Hommez, B. Pot, and F. Haesebrouck.** 1994. Identification and composition of the Streptococcal and Enterococcal flora of tonsils, intestines and feces of pigs. Journal of Applied Bacteriology **77:**31-36.
[61] **Li, F., M. A. J. Hullar, Y. Schwarz, and J. W. Lampe.** 2009. Human gut bacterial communities are altered by addition of cruciferous vegetables to a controlled fruit- and vegetable-free diet. Journal of Nutrition **139:**1685-1691.
[62] **Mroczynska, M. and Z. Libudzisz.** 2010. eta-glucuronidase and beta-glucosidase Activity of Lactobacillus and Enterococcus Isolated from Human Feces. Polish Journal of Microbiology **59:**265-269.
[63] **Nugon-baudon, L., S. Rabot, O. Szylit, and P. Raibaud.** 1990. Glucosinolates Toxicity in Growing-Rats - Interactions with the Hepatic Detoxification System. Xenobiotica **20:**223-230.
[64] **Rabot, S., L. Nugonbaudon, P. Raibaud, and O. Szylit.** 1993. Rapeseed Meal Toxicity in Gnotobiotic-Rats - Influence of a Whole Human Fecal Flora or Single Human Strains of Escherichia-Coli and Bacteroides-Vulgatus. Brit J Nutr **70:**323-331.
[65] **Oginsky, E. L., A. E. Stein, and M. A. Greer.** 1965. Myrosinase Activity in Bacteria as Demonstrated by Conversion of Progoitrin to Goitrin. Proceedings of the Society for Experimental Biology and Medicine **119:**360-4.
[66] **Oh, P. L., A. K. Benson, D. A. Peterson, P. B. Patil, E. N. Moriyama, S. Roos, and J. Walter.** 2010. Diversification of the gut symbiont Lactobacillus reuteri as a result of host-driven evolution. ISME Journal **4:**377-387.
[67] **De Angelis, M., S. Siragusa, M. Berloco, L. Caputo, L. Settanni, G. Alfonsi, M. Amerio, A. Grandi, A. Ragni, and M. Gobbetti.** 2006. Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Research in Microbiology **157:**792-801.
[68] **Evangelopoulou, G., S. Kritas, A. Govaris, and A. R. Burriel.** 2014. Pork Meat as a Potential Source of Salmonella enterica subsp arizonae Infection in Humans. Journal of Clinical Microbiology **52:**741-744.
[69] **Maskell, I. and R. Smithard.** 1994. Degradation of glucosinolates during *in vitro* incubations of rapeseed meal with myrosinase *(EC*3.2.3 1) and with pepsin *(EC* 3.4.23.l)-hydrochloric acid, and contents of porcine small intestine and caecum. British Journal of Nutrition **72:**455-466.
[70] **Oerlemans, K., D. M. Barrett, C. B. Suades, R. Verkerk, and M. Dekker.** 2006. Thermal degradation of glucosinolates in red cabbage. Food Chemistry **95:**19-29.
[71] **Lindahl, T.** 1974. N-Glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. Proceedings of the National Academy of Sciences of the United States of America **71:**3649-3653.
[72] **Saavedra, M. J., C. S. P. Dias, A. Martinez-Murcia, R. N. Bennett, A. Aires, and E. A. S. Rosa.** 2012. Antibacterial effects of glucosinolate-derived hydrolysis products against *Enterobacteriaceae* and *Enterococci* isolated from pig ileum segments. Foodborne Pathogens and Disease **9:**338-345.

CHAPTER IV

Validation of a quantitative method using liquid chromatography coupled to multiple mass spectrometry for thiouracil in feedstuffs used in animal husbandry

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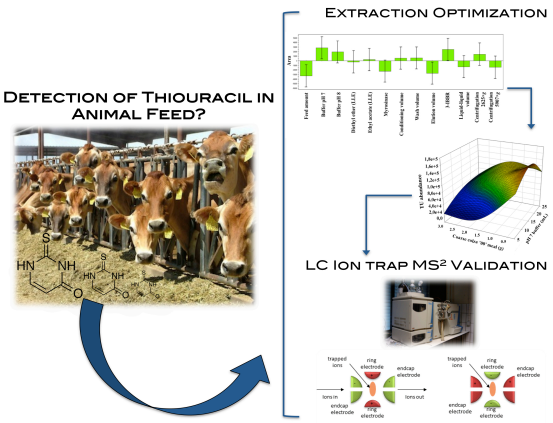
ABSTRACT

The use of thyreostatic drugs, like thiouracil (TU), in animal production has been banned for over three decades by the European Union, due to potential teratogenic and carcinogenic effects of its residues upon human consumption. Besides, thyreostats induce water retention in livestock, causing fallacious weight gain and deterioration of meat quality. Development of more competent analytical methods gave rise to sporadic TU detection in urine of untreated animals, questioning the actual synthetic origin TU. Research showed that TU can be formed upon digestion of Brassicaceae feeds *in vivo* and *in vitro*, which called for a means of differentiation between endogenous formation of TU and illicit administration. Therefore, in the present study, a routinely applicable LC ion trap MS^2 method for TU analysis in animal feeds was optimized and validated, according to CD 2002/657/EC. A fractional factorial Placket-Burman design was used to optimise the extraction procedure for TU from Brassicaceae and non-Brassicaceae feeds. This resulted in the discrimination of 5 influential factors (amount of feed, myrosinase, pH 7 buffer, 3-iodobenzyl bromide and elution solvent), for which the most optimal conditions were perfected. The limit of quantification for TU amounted 0.5 ng g^{-1}. The individual recoveries for TU ranged between 90.9 and 99.7%. Good results for repeatability and intra-laboratory reproducibility (RSD%) were observed, i.e. respectively ≤ 6.0% and ≤ 5.2% for TU. Excellent linearity was proven based on determination coefficient (R^2 ≥ 0.99) and lack-of-fit test (*F*-test, α=0.05).

Subsequently, a selection of feeds sampled during European national monitoring campaigns were evaluated with the present method showing concentrations ranging from 0.32-20.60 ng g^{-1}, demonstrating the relevance of the method in the analysis of TU from animal feeds.



Keywords: thiouracil, animal feed, Plackett-Burman design, Liquid Chromatography ion trap MS ($LC\text{-}MS^2$)

1. INTRODUCTION

Thyreostats, like thiouracil (TU), are thyroid-inhibiting compounds of synthetic origin, which once administered cause a decrease of thyroid hormone production. Because of their association with illegal fattening in animal production and the subsequent risks of potential teratogenic [1-3] and carcinogenic effects (2B substance) [4] of their residues for public health, thyreostats have been banned in the European Union (EU) for over three decades [5]. More recently, the exclusively synthetic origin of thyreostats was questioned due to the sporadic, but systematic detection of low-level concentrations in livestock urine during national control campaigns all over the EU [6-8], facilitated by better analytical methods [9-11] able to quantify concentrations below the set minimum required performance limit (MRPL) of 100 µg L^{-1}. A first *in vivo* trial performed by Pinel *et al*. (2006) [12] demonstrated the endogenous origin of urinary TU resulting from digestion of glucosinolate-rich crops belonging to the Brassicaceae family. Consequently, the European Union reference laboratories (EURL) issued a reference document [13] suggesting low-level TU concentrations below 10 ng g^{-1} were most probably originating from feed and should thus be regarded as compliant.

Brassicaceae crops have been widely used in animal production because of their inexpensive high protein content. Nevertheless, these crops are also known for their high content in glucosinolates, which are secondary plant metabolites involved in the plant's defence against tissue damaging pests [14]. The catabolism of glucosinolates as a consequence of cell disruption (e.g. chewing) followed by interaction with the plant-derived myrosinase (ß-thioglucosidase, E.C 3.2.1.147) [15], hydrolysis or bacterial digestion in the gut [16-19], results in various degradation products (oxazolidine-thiones, nitriles, epithionitriles thiocyanates, isothiocyanates, thiourea) depending on specific pH conditions [20,21].

Some of these glucosinolate-derived decomposition products own natural goitrogenic properties thereby negatively affecting the thyroid function, e.g.: oxazolidine-thiones (goitrin), thiocyanates and thiourea [22]. Others, like isothiocyanates, are responsible for bitterness [23,24], whereas nitriles can cause behaviour abnormalities (alteration in reflex behaviour, increased locomotor activity, circling, head twitching and occasional backward pedalling) [25]. Even more adverse effects can be caused by glucosinolate metabolites including besides goitrogenicity [22,26-28] also mutagenicity, hepatotoxicity and nephrotoxicity [25,29]. Negative effects of glucosinolates on animal production and growth are proportional to their dietary concentration [30] and therefore intimately related to their

exploitability in the feed industry [31]. Subsequently, this has led to the minimisation of glucosinolate content of Brassicaceae crops through genetic and breeding means with development of the '00'-type plants, which have a low glucosinolate content (max. of 25 µmol g^{-1} at a moisture content of 9%) [32]. Additionally, TU has been detected in the urine of untreated animals (porcine, bovine, ovine, canine, equine) and humans, who were not subjected to a Brassicaceae enriched diet. This illustrates the ambiguity of the problem since TU can be present, regardless of TU treatment or a Brassicaceae enriched diet [15].

The ability to determine whether the feed itself, Brassicaceae enriched or not, may contain endogenous TU levels, prior to oral consumption and gastro-intestinal digestion, would be a valuable tool to elucidate causal factors for the presence of endogenous TU. To the best of our knowledge, no such validated and routinely applicable method has been described, based upon a statistically optimised extraction procedure for thiouracil in both Brassicaceae and non-Brassicaceae feedstuffs.
Therefore, the focus of the present study was to optimize a feed extraction procedure for TU using a fractional factorial design (Plackett-Burman) to elucidate the factors influencing the extraction, which subsequently could be optimized in a Central Composite Face (CCF) design. Moreover, this allows a quicker and more cost-effective optimization, compared to one-at-the-time experiments. Validation of the ion trap mass spectrometrical method (LC ion trap MS^2 detection) was performed according to CD 2002/657/EC [33]. Finally, its practical applicability was evaluated through analysis of several feed samples, which were sampled during the Irish national control campaign (2012-2013).

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The chemical standard 2-thiouracil (TU) was obtained from Sigma-Aldrich (St. Louis, USA). The deuterated internal standard for TU, 6-propyl-d_5-2-thiouracil (PTU-d_5) was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Stock solutions of the chemical standards were prepared in methanol at a concentration of 200 ng μL^{-1}. Working solutions were diluted in methanol to 1 ng μL^{-1} for PTU-d_5 and 1 ng μL^{-1}, 0.1 ng μL^{-1} and 0.01 ng μL^{-1} for TU. Solutions were stored in dark glass recipients at 4 °C. Reagents were of analytical grade (VWR International, Merck, Darmstadt,

Germany) when used for extraction and purification steps, and of Optima® LC-MS grade for LC-MS application (Fisher Scientific UK, Loughborough, UK), respectively. The derivatisation reagent, 3-iodobenzyl bromide (3-IBBr, Sigma-Aldrich, St-Louis, USA), was prepared extemporaneously (2 mg mL^{-1} methanol). The phosphate buffer, made up of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 (Merck, Darmstadt, Germany) in deionized water, was prepared and adjusted to pH 8. The phosphate buffer pH 7 was made up in a similar way with 0.5 M $Na_2HPO_4.H_2O$ and 0.5 M KH_2PO_4.

2.2. Feed samples

The animal feeds selected for optimisation were affiliated to the Brassicaceae family, as they are known for their high glucosinolate content. However, nowadays only '00' varieties are used for consumption (human-animal) due to the anti-nutritional effects they may cause. Therefore, a '00' rapeseed derivate was chosen as the Brassicaceae representative. Additionally, a non-Brasscicaceae cattle feed, which consisted mainly of 40% linseed cake, and 20% of rolled barley and spelt (dinkel), was used as a blank. The 22 feed samples from the Irish national control plan campaign are presented in Table IV.3. Feeds were homogenised using a blender starting from a 100 g homogenous subsample and subsequently stored in dry and dark conditions at 4 °C.

The myrosinase enzyme was extracted from white mustard seed (*Sinapis alba* L.) supplied by a local supplier (Mostaardfabriek Oud Huis Ferdinand Tierenteyn bvba., Deinze, Belgium), referred to as 'Myro A'. To evaluate the activity of the extracted myrosinase, it was matched to a previous report by Vanden Bussche *et al.* (2011) [15], which compared the activity of extracted myrosinase from mustard seeds with synthetic myrosinase, and saw no significant differences. Moreover a mustard seed subsample from the supplier of Vanden Bussche *et al.* (2011) [15], was also investigated ('Myro B').

2.3. Optimization of sample extraction and purification

A statistical model design was used to optimize the analytical extraction procedure of thyreostats in animal feed. The dependent variables that might significantly affect the extraction efficiency were screened with a Plackett-Burman design, which is a fractional factorial design. Further optimization of the selected variables was performed with Response Surface Modeling (RSM) (i.e. Central Composite Face design (CCF)). In general, a fractional factorial design is a good screening approach since the amount of variable scenarios (trials) produced by such a model, is

significantly lower than that for full factorial designs. Unlike, full x^k factorial designs, which require all combinations of x versions of each of k variables, a fractional factorial design only needs x^{k-1} [34].

The variables that might influence the extraction of thyreostats from animal feed were selected from previous scientific work on the extraction of thyreostats from various matrices [9,15,35-39]. First a Plackett-Burman design investigated the extraction protocol based on non-Brassicaceae cattle feed fortified with TU. Secondly, coarse colza '00' meal belonging to the Brassicaceae was evaluated without any TU fortification, thus evaluating the effect of myrosinase addition on the extraction procedure of TU. The 9 quantitative and 2 qualitative factors and their respective levels are presented in Table IV.1.

For each feed, 12 experimental runs plus 3 central value runs sufficed to specify a particular combination of settings for the different variables included in the design. After performing the prescribed scenario on non-Brassicaceae and Brassicaceae feeds, the main effects of each variable were calculated. After a proven statistical significant analysis ($p < 0.05$), the critical variables (feed, 3-iodobenzyl bromide, buffer pH 7, elution volume) were subjected to further optimization along with the myrosinase variable since it had, as the above variables, a Variable Importance for Projection (VIP) value > 1 by response surface methodology. To this extent, a Central Composite Face (CCF) design was used, where star points, which represent runs where all but one of the factors are set at their mid-levels, are placed on the faces of the design matrix. This is a full two level full factorial approach enabling evaluation of interactions between levels. If a factor proved to be non-critical, the best level, according to its effect, was chosen. Generally, this will be the least time-consuming, cheapest or most result-effective level of the factor, which is then built into the protocol [40]. Based upon these results a final protocol could be assembled for thyreostat extraction from feed.

The software program Modde 5.0 (Umetrics, Umea, Sweden) was used to build the experimental design matrix and carry out data analysis. The parameters were inserted into the program and depending on the purpose of the analysis (screening or RSM), the most adequate model was proposed by the program. Evaluation of the models was done through a one-way variance analysis (ANOVA) test. Significance was proven when *p*-values were smaller than 0.05.

Table IV.1: Plackett-Burman factors with their specific lower and upper limits for the optimization of the extraction of thiouracil from animal feed, respectively applied to non-Brassicaceae and Brassicaceae (coarse colza '00' meal) feeds.

PB design	Variables	Level 1	Level 2
Qualitative	Liquid-liquid solvent	Diethyl ether	Ethyl acetate
	Centrifugation speed	2625 × *g*	5907 × *g*
Quantitative	Feed	0.5 g	2 g
	Buffer pH7	5 mL	15 mL
	Myrosinase	0 mL	0.5 mL
	Buffer pH 8	5 mL	15 mL
	Derivatisation (3-IBBR)	100 µL	1000 µL
	Liquid-liquid volume	5 mL	10 mL
	Conditioning volume	10 mL	20 mL
	Wash volume	5 mL	10 mL
	Elution volume	5 mL	10 mL

2.4. Myrosinase preparation

Myrosinase enzyme used in the optimisation of the extraction protocol was adopted from Wrede (1941) [41] as mentioned in Vanden Bussche *et al.* (2011) [15]. Briefly, 5 g of mustard seeds were grounded with 30 mL of ultrapure water. Subsequently, the homogenous suspension was stirred for 30 min at room temperature and centrifuged for 10 min at 12000 × *g*. To the supernatant an equal volume of 90% ethanol was added dropwise, whilst vortexing. After centrifugation the precipitated plaque was washed dropwise with 10 mL of 70% ethanol and centrifuged. The remaining precipitation was then redissolved in 5 mL of ultrapure water, containing approximately 20 mg mL^{-1} of the enzyme [42].

2.5. Control of myrosinase enzymatic activity

The enzymatic activity of myrosinase was checked prior to use in order to verify the activity was not significantly different from the activity described in Vanden Bussche *et al.* (2011) [15], which used this enzymatic procedure in the extraction of feed. Glucose was detected, as it is the by-product of the unimolecular reaction between myrosinase and the commercially available glucosinolate sinigrin (Sigma-Aldrich, St. Louis, USA). To this end, a buffer pH 7 was made up from 0.1 M ammonium acetate and 0.025 M ammonium bicarbonate and added with 34 mM of sinigrin. Then, in a first experiment 1.5 mL of buffer was incubated for 4 h at 37 °C with 0-0.5-1 mL of freshly made-up myrosinase solution (n=3) either from the 'Myro A' or 'Myro B' (from the supplier of Vanden Bussche *et al.* 2011 [15]) mustard seeds. The hydrolysis reaction

was stopped through boiling of the samples for 5 min and glucose was subsequently detected with HPLC-ELSD (high performance liquid chromatography coupled to evaporative light scattering detection) [15,43] with a gain factor of 2. Quantification was performed based on a buffer calibration curve (0, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 mg mL^{-1}).

The quality control showed glucose production was similar ($p > 0.05$) at 0.5 and 1 mL for both mustard seeds. The concentrations were in relation with previous results proving a sufficiently high enzymatic activity was present to extract TU from feed matrices [15].

A second experiment evaluated the effect of increasing the myrosinase amount on the detection of TU from coarse colza '00' meal. Myrosinase solution (0-0.5-1-1.5-3 mL) was added to the extraction prior to the 1 h sonication and subsequent overnight incubation [44]. The increasing myrosinase amount

s showed a significant ($p < 0.05$) decreasing trend for TU detection from feed.

Therefore, from these control experiments, the conclusion was drawn that the extracted myrosinase used in the extraction optimization was of good quality and had sufficient enzymatic activity [15]. Moreover, an increasing amount of myrosinase did not increase the detection of TU, therefore a 0-0.5 mL range of myrosinase solution was chosen in the PB evaluation for Brassicaceae and non-Brassicaceae feeds.

2.6. Final extraction protocol

Feed samples were prepared using the final protocol resulting from both Plackett-Burman designs on Brassicaceae and non-Brassicaceae feed. The extraction started with 10 mL of phosphate buffer (pH 7), which was added to 0.25 g of feed followed by vortexing, 1h of sonication and an overnight incubation at 37 °C with continuous rotation at 150 rpm. Next, samples were centrifuged at 10503 × *g* for 10 min and the supernatants was pored over a small amount of cotton wool placed into a funnel collecting the liquid into a 50 mL falcon tube. Hereupon, internal standard and 15 mL of phosphate buffer pH 8 were added along with 3000 µL of a methanolic derivatizating solution containing 3-iodobenzyl bromide (2 mg mL^{-1}). Samples were sonicated for 10 minutes and left to derivatise for 1 h at 40 °C in the dark. Afterwards the pH of the reaction mixture was adjusted to 3.6 (± 0.1) and 3 liquid-liquid extraction steps of 5 mL with diethyl ether were applied, collecting only the supernatant after each centrifugation step (4 min at 2625 × *g*). The latter phase was evaporated to dryness under a gentle N_2 stream (2 bar, 50 °C) and redissolved in 100 µL CH_2Cl_2 and 300 µL cyclohexane. Furthermore, solid phase extraction with silica cartridges (10 mL,

500 mg SI Isolute, Biotage, Uppsala, Sweden) was conditioned with 15 mL of cyclohexane, washed with 7.5 mL of cyclohexane and eluted with a 3 mL mixture of hexane/ethyl acetate 40/60 (v/v). Again samples were evaporated under N_2 and redissolved in 200 µL (or 160 µL, using standard addition) of methanol and 0.5% acetic acid (50/50), briefly vortexed and sonicated (5 min). Finally the extracts were centrifuged at 12304 × *g* for 10 min before transferring to plastic LC-MS vials.

2.7. Instrumentation

2.7.1. Liquid chromatography coupled to multiple mass spectrometry (LC-MS²)

Analysis of TU was achieved with a liquid chromatograph coupled to a linear ion trap mass spectrometer, which was adopted from Vanden Bussche *et al.* (2011) [15] and implemented with alterations, regarding gradient, interface gas flow, acquisition and divert valve time. A Finnigan Surveyor LC-system (Thermo Electron, San Jose, USA) was combined with a Symmetry C_{18} column at 30 °C (5 µm x 150 mm x 2.1 mm, Waters, Milford, Massachusetts, USA) running on a 0.5% acetic acid (A) and methanol (B) 50/50 solvent combination at 0.3 mL min^{-1}. The linear gradient was adapted over a 35 min run: A/B 50/50 for 3 min, increasing to 0/100 in 20 min, and finally re-equilibrating for 10 minutes at 50/50. The linear ion trap mass spectrometer LTQ (Thermo Electron, San Jose, USA) was fitted with a heated electrospray ionization probe (HESI) operating in the negative ion mode and positioned on 0.5; C; 0 (front-to-back position (µm); probe depth (probe depth line); side-to-side position (+1 to -1 marks)). Applied working conditions were as follows: source voltage at 5 kV; capillary voltage at -50 V; tube lens voltage at -128.04 V; vaporizer and capillary temperature at 250 °C and 275 °C; sheath and auxiliary gas at 30 and 5 arbitrary units (a.u.), respectively. Sweep gas was increased to 2 to minimise interference and preserve the device. Data acquisition was now performed over the entire run and the divert valve was switched at 4 (0-4-20-30 min) different occasions decreasing soilage of the MS. The transitions of TU ($[M-H]^-$ = 343 *m/z*) were obtained, applying a collision energy of 44 eV with 182, 215, 309 (*m/z*) as the main product ions. The internal standard PTU-d_5 ($[M-H]^-$ = 390 *m/z*) required 30 eV to fragment into its main product ions 127, 262, 356.

2.7.1.1. Standard addition

Quantification of TU in the Irish feed samples was performed using the standard addition approach as described in Commission Decision 2002/657/EC [33]. Each sample was divided over 2 vials with analogous mass (m) and volume (V). One

aliquot, the unknown, was added with a mixture of 0.5% acetic acid and methanol 50/50 (V_A) and the other, the known, was enriched with an equal amount of analyte (TU) (V_{known}). The concentration of the addition solution was previously determined by analysing TU in feed samples and fitting these in a calibration curve in eluens. This approximate estimation of the TU concentration was then spiked to the aliquot of each sample (ρ_A) giving rise to the area ratio of χ_{known}. Finally, quantification was established through evaluation of the area ratios of the known and unknown samples using the standard addition approach [15].

Quantification was established using the following formula: $C_{unknown} = \chi_{unknown} V_{unknown} \rho_A V_A / (\chi_{known} V_{known} m_{unknown} - \chi_{unknown} V_{unknown} m_{known})$, with $m_{unknown} = m_{known}$, $V_{unknown} = V_{known}$ and $C_{unknown} = \chi_{unknown} \rho_A V_A / (\chi_{known} - \chi_{unknown})$. In this formula, C, ρ are concentrations, χ is the area ratio, V is the volume, m is the mass and A is identified analyte (TU).

2.7.1.2. Quality assurance

Preceding the LC-MS^2 analysis a standard mixture of the target compound and the internal standard were injected to check the operational conditions of the LC-MS^2 device. Identification of TU was based on retention time relative to the internal standard, and the ion ratios of the product ions according to Commission Decision 2002/657/EC [33].

2.8. Validation

Once the extraction procedure had been optimised the analytical method was validated in order to show the applicability and robustness. To this extent, a validation protocol in accordance with the CD 2002/657 [33] was adopted, with slight alterations using limits of detection (LOD) and quantification (LOQ) instead of CC_α and CC_β, which has been a common practice for the quantitation of group A substances in animal feed [45-47]. Moreover, animal feed is strictly speaking not included in food consumption for which the commission directive was initially developed [33]. Subsequently, non-Brassicaceae animal feed (n=20) was analysed to check the ruggedness. The feed samples were subsequently fortified in order to determine the specificity of each peak based on retention time and signal amplitude increase according to the fortification level (n=3). For the validation, blank feed samples (6 per level) were fortified with 3 different concentrations (TU: 5-10-15 ng g^{-1}). The quantification was based on matrix matched calibration curves consisting of 8 fortification levels (2.5-5-7.5-10-12.5-15-20-25 ng g^{-1}). The linearity of the curves was evaluated through the coefficient of determination (R^2), which should be ≥ 0.99 [33]

and the lack-of-fit (LOF), which is a well-known linearity evaluator [48]. The LOF was assessed for TU in animal feed, taking into consideration the deviation of linearity of the datasets by building an univariate linear regression model (SPSS 21, IBM, USA) with the calibration curve replicates (n=3), setting concentration as independent variable and the respective area ratios (e.g. area compound/area respective internal standard) as dependent variable. PTU-d_5 was chosen as an appropriate internal standard [10].

2.9. Data handling

All LC-MS^2 data processing was performed with XCalibur 2.0.7 (Thermo Fisher Scientific, San Jose, CA, USA). Linearity evaluation was established through lack-of-fit analysis (*F*-test, α=0.05) of the calibration curves in SPSS 21 (IBM, USA). Statistical testing (Student's-t-test) was also carried out in SPSS 21 to assess significance ($p < 0.05$). Normality and equal variance were prerequisites. All statistical evaluation in relation to the designs was performed with Modde 5.0 (Umetrics, Umea, Sweden).

3. RESULTS AND DISCUSSION

3.1. Statistical extraction optimization of thiouracil from animal feeds

The strength of fractional designs is that they provide a significant time and effort reduction without losing valuable information and still produce comparable results to the laborious full factorial design (e.g. 16 trials versus 288 trials) [49]. The Plackett-Burman (PB) design [50] in particular, is useful to distinguish from a large number of variables those variables that might affect the response of the component(s) of interest. PB consists of a two-level design (2^{k-1}), which investigates $^{k-1}$ variables, with k runs, where k is a multiple of 4. The amount of runs will generally be augmented with 3 central points to obtain informative data about the range between the upper and lower limit of the variables [51]. Since fractional factorial designs, like PB, give little to no insight in possible interactions between the factors, implementation of a sensitivity analysis by means of a full factorial design (e.g. Central Composite Designs) should be set up with the factors that proved to be critical in the fractional factorial design [40,52]. Based on coefficient plots on the one hand and variable importance plots on the other, the effects of both feeds on TU extraction (Figure IV.1 to IV.3) could be evaluated.

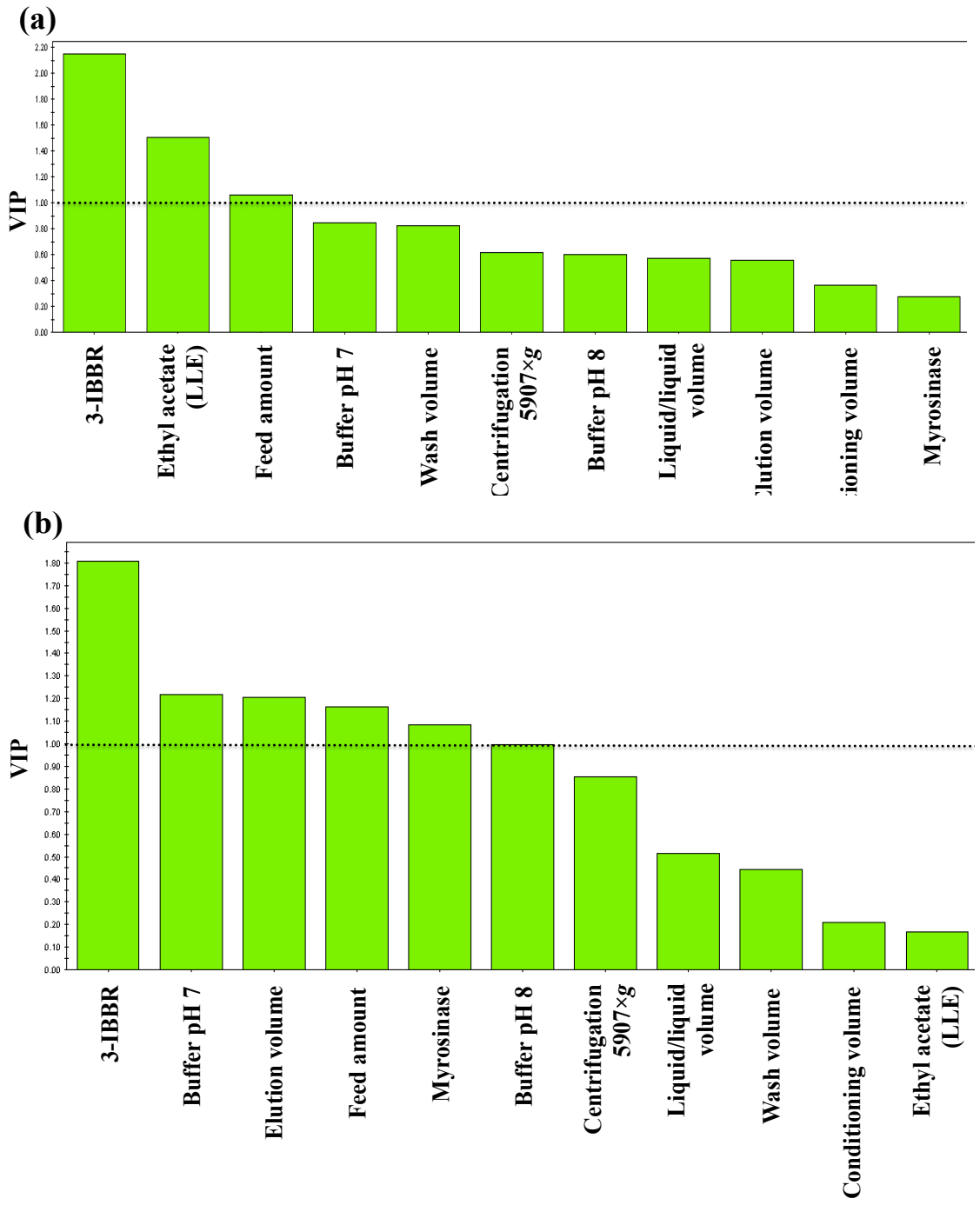


Figure IV.1: (a) Variable importance plot from the Plackett-Burman design in non-Brassicaceae feed, showing feed amount, ethyl acetate and 3-iodobenzyl bromide were important factors (VIP>1) in the extraction of thiouracil (TU) from feed. (b) Variable importance plot of TU in non-fortified Brassicaceae feed (coarse colza '00' meal), showing variable importance for projection values > 1 for 3-iodobenzyl bromide, pH 7 buffer, elution volume, feed and myrosinase. These 5 factors were included in the response surface modelling optimization (LLE: Liquid-liquid extraction).

In the first PB experiment blank non-Brassicaceae feed was used, which was fortified with TU. This design only resulted in non-significant ($p > 0.05$) effects, but from the VIP some effects could be observed (Figure VI.1-a). In general the following rule of thumb can be applied: Y: VIP > 1.0 (highly influential), 0.8 < VIP < 1.0 (moderately influential), VIP < 0.8 (less influential) and VIP < 0.5 ('unimportant') [51,53]

In a second experiment with the same PB design, but with a non-fortified Brassicaceae feed 4 significant factors ($p < 0.05$) were found (Figure IV.2): feed (negative effect), buffer pH 7 (positive effect), elution volume (negative effect) and 3-iodobenzyl bromide (positive effect). Myrosinase and buffer pH 8 were not significant ($p < 0.05$), exerting respectively a negative and positive effect.

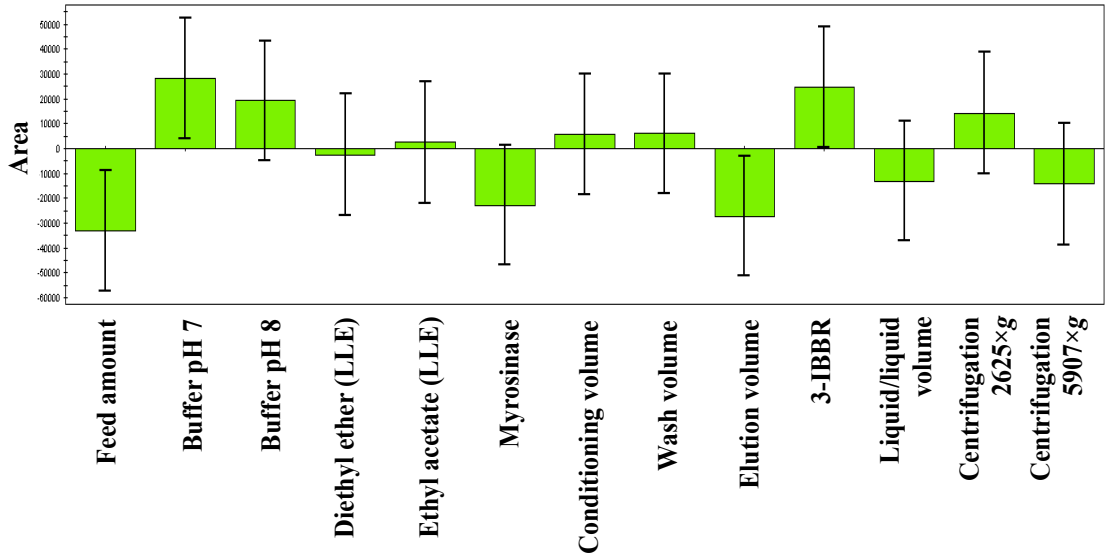


Figure IV.2: Coefficient plot for thiouracil in non-spiked Brassicaceae feed (coarse colza '00' meal). Significant factors being feed (negative effect), pH 7 (positive effect), elution volume (negative effect), 3-IBBR (positive effect). Myrosinase (negative effect) and pH 8 buffer (positive effect) were almost significant (LLE: Liquid-liquid extraction).

Next, a sensitivity analysis of the critical PB factors in Brassicaceae feed was conducted by means of a CCF (Central Composite Face) full factorial design to gain insight in the possible interactions between factors [40,52]. Based on the VIP results (Figure IV.1-b), besides the above 4 significant factors, also myrosinase (VIP > 1) was included in the subsequent optimization of the factors with CCF design through

Response Surface Modelling (RSM) (Figure IV.3). Typically in a CCF, all scenarios per factor (feed amount, buffer pH 7, elution volume, 3-iodobenzyl bromide, myrosinase) are evaluated at low-, high- but also at mid-level ranges. These levels are the star-points of the design and are set at the center of each face of the factorial space and chosen within the range of each factor [51]. Evidently, because the number of scenarios becomes equal to 2^k, including a lot of parameters (k) becomes less feasible. Hence, a screening model (PB) was applied to the variables in this study, to limit the number of remaining variables for further optimization in the full factorial model with Response Surface Modeling (CCF). Finally, the amount of feed 0.25-3 g, pH 7 buffer 5-25 mL, 3-iodobenzyl bromide 250-3000 µL, myrosinase 0-0.5 mL and the elution volume 2.5-15 mL were set at different levels based on the PB results and evaluated within the CCF design. This showed 5 significant factors ($p < 0.05$) in its coefficient plot, i.e.: feed amount (negative effect), myrosinase (negative effect), elution volume (negative effect), 3-iodobenzyl bromide (positive effect) and buffer pH 7 (positive effect). Two significant negative interactions were also uncovered for 3-IBBR towards elution volume and myrosinase, respectively. Consequently, these factors were optimized based on the RSM plots and the resulting parameters were built into the extraction procedure, respectively: 0.25 g of feed, 15 mL of pH 7 buffer, 3000 µL of 3-iodobenzyl bromide, 3 mL of elution solvent and no addition of myrosinase.

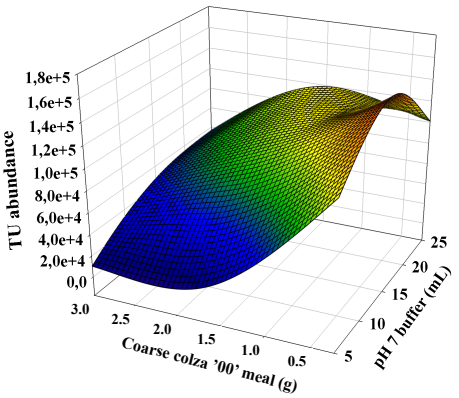


Figure IV.3: Example of a response surface model plot for the influence of buffer pH 7 and the amount of Brassicaceae feed (coarse colza '00' meal) on the extraction of thiouracil, resulting in the optimal conditions: 0.25 g of feed and 15 mL of buffer pH 7.

In conclusion, the preference for the applied low feed amounts is comprehensible since matrix interferences increase when higher feed amounts are used and if the detection method is sensitive enough this should not be an issue. Matrix interferences causing high background noise in feeds, like feeding cabbage and meal with 30% of rapeseed '00' have been reported, which even when fortified with 10 µg kg^{-1} TU, still

yielded a non-detectable signal with LC-MS^2 [15]. Since the addition of myrosinase proved not requisite for TU detection, the influence of incubation and phosphate buffer pH 7 and to al lesser extent pH 8 [9,15], are of increasing importance to the extraction of TU. Another pivotal factor is the high volume of derivatisation agent, since too little will cause the present TU to stay underivatised, which makes it undetectable to the MS analyser. Overall, the adaptations made to the extraction procedure clearly optimized the detectability of TU in animal feed.

3.2. Validation study

Upon optimization of the extraction and analysis, the method was validated according to the criteria specified in CD 2002/657/EC for quantitative confirmation [33]. An appropriate deuterated internal standard, PTU-d_5, was chosen for its capability to anticipate fluctuations in the signal intensity upon extraction [7,10,15]. The validation results are presented in Table IV.2. Spiking concentrations were based on the recommended concentration (RC) of 10 µg kg^{-1} by the European reference laboratories [13].

3.2.1. Specificity and selectivity

The chromatograms of 20 blanks indicated that no other matrix substances significantly interfered with the signals of the compound of interest, since signal-to-noise ratios of the neighbouring background chromatographic peaks, if present, were never higher than 3 (Figure IV.4-a and IV.4-b). When samples were fortified, a significant increase of the peaks of interest at the expected retention times with a substantial augmentation in area and signal-to-noise ratio were observed, thereby proving its selectivity for the analytes of interest. In accordance with CD 2002/657/EC [33], analytes were identified on the basis of their relative retention time, i.e. the ratio of the retention time of the analyte to that of the internal standard. As a result, the developed method was found to be specific and selective for TU in the presence of matrix compounds.

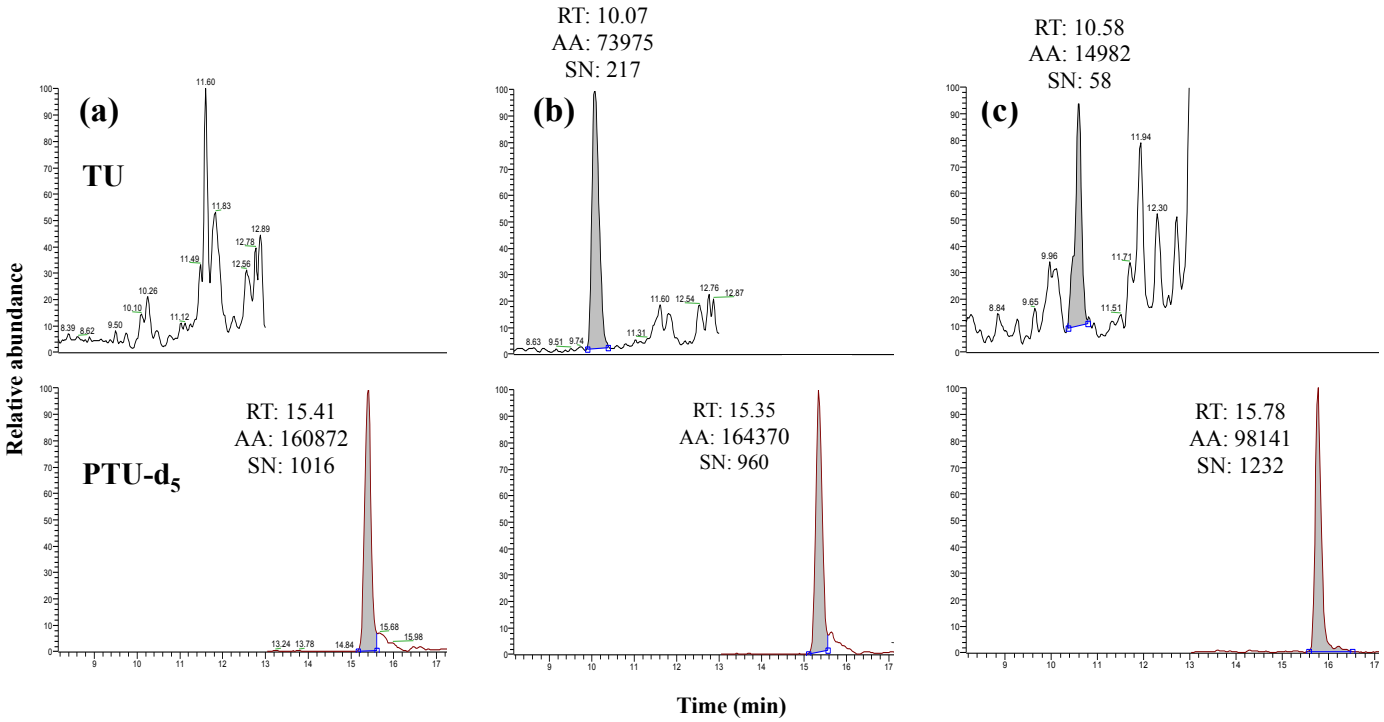


Figure IV.4: Chromatographic representation of a blank (a) and a spiked (2.5 ng g^{-1}) (b) non-Brassicaceae feed sample, showing retention time (RT), peak area (AA) and signal-to-noise ratio for thiouracil (TU) and its internal standard (PTU-d_5). The chromatogram of a TU spiked non-Brassicaceae feed sample at the LOD 0.2 ng g^{-1} (c).

3.2.2. *Linearity*

9-point calibration curves were elaborated in blank feed to evaluate the linearity of the developed method for TU detection from animal feed. The samples were fortified with concentrations reported under *2.8 Validation*. Linearity performed well since determination coefficients (R^2) were all ≥ 0.99 and commensurable with literature findings for TU analysis in fodder [54-56]. Furthermore, the regression model equations resulting from lack-of-fit [48] were reported to be all linear (*F*-test; α=0.05).

3.2.3. *Precision*

The precision of the developed analytical method was evaluated through determination of repeatability and within-laboratory reproducibility. Both validation parameters were evaluated by calculating their relative standard deviations (RSD%). To assess the repeatability, three series of six replicates at three different fortification levels in blank animal feed, were analyzed by the same operator under repeatable conditions (Table IV.2). The calculated RSD% values were excellent since values were well below 15% compliant with CD 2002/657/EC [33] and ranged from 5.4-6.0%. For

intra-laboratory reproducibility four series of six replicates of samples, at three fortification levels analysed by different operators on different days were evaluated. The calculated intra-laboratory RSD% ranged from 4.7-5.2 %, which indicates very good within-laboratory reproducibility, with reference to the 20% upper limit set by the CD 2002/657/EC [33]. In literature reports may be lower but these are based on µg g^{-1} concentrations, e.g. within day RSD% of 1.9-2.6% at 0.6-1-5 µg TU g^{-1} and between day 2.6 RSD% in maize flour [55] and 3-7 repeatability RSD% at 20-200 µg TU g^{-1} [54]. Kong *et al.* (2009) [56] based its precision evaluation on standard solutions reaching a 1.9 RSD% for reproducibility and 3 RSD% inter-day precision. Only recently a method based on LC-triple quadrupole analysis for thyreostat analysis in feedstuffs was published, but showed high repeatability and within-laboratory reproducibility (up to 19.6 RSD%) [57].

3.2.4. Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) are the lowest levels at which a compound can be, respectively, identified or quantified with a signal-to-noise ratio (S/N) of respectively, ≥3 and ≥10. For TU the detection and quantification limits were theoretically calculated based on 9-point calibration curves in matrix, correlating concentrations to signal-to-noise values [58]. An LOD of 0.2 ng g^{-1} and an LOQ of 0.5 ng g^{-1} were deducted and confirmed using spiked matrix-matched samples (Figure IV.4-c). Even though validation studies of mass spectrometrical techniques for the analysis of similar components in animal feed are scarce or non-existing, some studies did aim to develop methods for thyreostats and analogues, using other techniques. One method resorted to micellar electrokinetic chromatography (MEKC) for the detection of thyreostats in animal feed by diode array reaching LODs of 0.4-0.6 µg g^{-1} of fodder and 4% peak area repeatability [54]. Thereupon a capillary electrophoresis combined with laser-induced fluorescence detection method showed recoveries at µg g^{-1} levels [55], which was enhanced using an electrochemical detector achieving LODs ranging from 7.6-25 ng g^{-1} for various thyreostats in feed (18 ng g^{-1} TU; 25 ng g^{-1} PTU) [56]. Mass spectrometrical methods mentioning similar LODs for the analysis of TU and analogues specifically for animal feed matrices have not yet been reported to the best of our knowledge. Therefore the obtained results are undeniable satisfactory and even when compared to other mass [57] and non-mass spectrometrical technologies [54-56] for the detection limit of the thyreostats at hand in animal feed, the present method performs better.

3.2.5. Mean recovery

As no certified reference material was available, trueness was determined as the mean corrected recovery by using fortified blank animal feed samples. To this extent, three fortification levels were considered with six replicates for each level. The present recoveries ranged from 90.9 to 99.7% for TU. Results were found satisfactory since the lowest mass fraction (10-100 ng g^{-1}) according to the CD 2002/657/EC [33] requires mean recoveries between 80-120%. Reported mean recoveries ranged between 78-81% at 0.6-1-5 µg TU g^{-1} [55] and in another case mean recoveries ranged between 85-105% for various thyreostats at a concentration of 0.3 µg g^{-1} without further specifications [56]. In a singular report on LC-triple quadrupole analysis in animal feedstuff recoveries only ranged between 77.7-86.9% for 5-10-15 ng TU g^{-1} [57].

Table IV.2: Overview of the methods performance for the detection of thiouracil in non-Brassicaceae feed (n=128).

Analyte	**LOD (ng g^{-1})**	**LOQ (ng g^{-1})**	**Nominal concentration (ng g^{-1})**	**Recovery (%)**	**Precision**	
					Repeatability RSD (%)	**Within-lab. Reprod. RSD (%)**
Thiouracil	0.2	0.5	5	90.9±7.4	6.0	5.2
			10	98.9±4.8	5.4	5.0
			15	99.7±1.5	5.7	4.7

3.3. Thiouracil analysis of various animal feeds

To prove the applicability of this newly optimized method, various animal feeds (n=22) from the Irish control plan and coarse colza '00' meal (n=1) were analysed for TU with the newly optimized method (Table IV.3). TU concentrations in the feeds were identified according to the identification point (IP) policy of 4 IPs described by CD 2002/657/EC [33].

Table IV.3: Concentration (ng g^{-1}) of thiouracil detected in various types of animal feeds using the newly optimized and validated LC-MS^2 method (n.d.: not detected).

Animal Feed	**ng g^{-1}**
Vitamin mineral yeast mix	n.d.
Beef ratio creamery	11.93
Barley and propionic acid	9.35
Optigen 11 (non-protein-nitrogen)	n.d.
Distillers dried grains	n.d.
Cattle meal (rolled barley, rolled wheat, soya hulls, citrus pulp pellets, wheat distillers dried grains, sugar cane molasses, extracted soya bean, toasted extracted rapeseed, beans, vitamins and trace elements)	3.76
Beef finisher meal	20.60
Oil seed rape	10.27
Beef finisher	5.19
Beef feed (silage, straw, concentrate)	n.d.
Maize meal	0.32
Mix of soya hulls, rape seed & barley	13.14
Barley	5.35
Ground barley & wheat	6.79
Soya Hulls	11.56
Mineral feed	n.d.
Beef finisher	9.98
Maize meal	2.19
Beef meal	8.21
Maxammon Grain preservative	n.d.
Urea-part of Maxammon Grain preservative	n.d.
Beef meal	n.d.
Coarse colza '00' meal	7.04

The results show above 10 ng g^{-1} TU concentrations, not surprisingly, in the Brassicaceae oil rapeseed, but also in soya hulls and composed beef ratios (creamery, finisher meal) with unknown composition. Soybean meal is one of the major ingredients in swine rations and has also been reported to possess goitrogenic properties [59], which are not glucosinolate-based. In Brassicaceae crops, seeds

have been shown to contain progoitrin as the main glucosinolate [60]. In their leaves, two different glucosinolate profiles have been reported depending on the type of Brassica crop: forage and root vegetable crops showed high levels of progoitrin, whereas glucobrassicanapin is the main glucosinolate for oil seed and leafy crops [60]. Even though a clear trend cannot be distinguished, these results do show that TU can be detected in Brassicaceae feeds, as well as, non-Brassicaceae feeds, which has not been reported yet. Moreover, the applicability of the method in various feeds with different consistencies and compositions has been demonstrated (Figure IV.5).

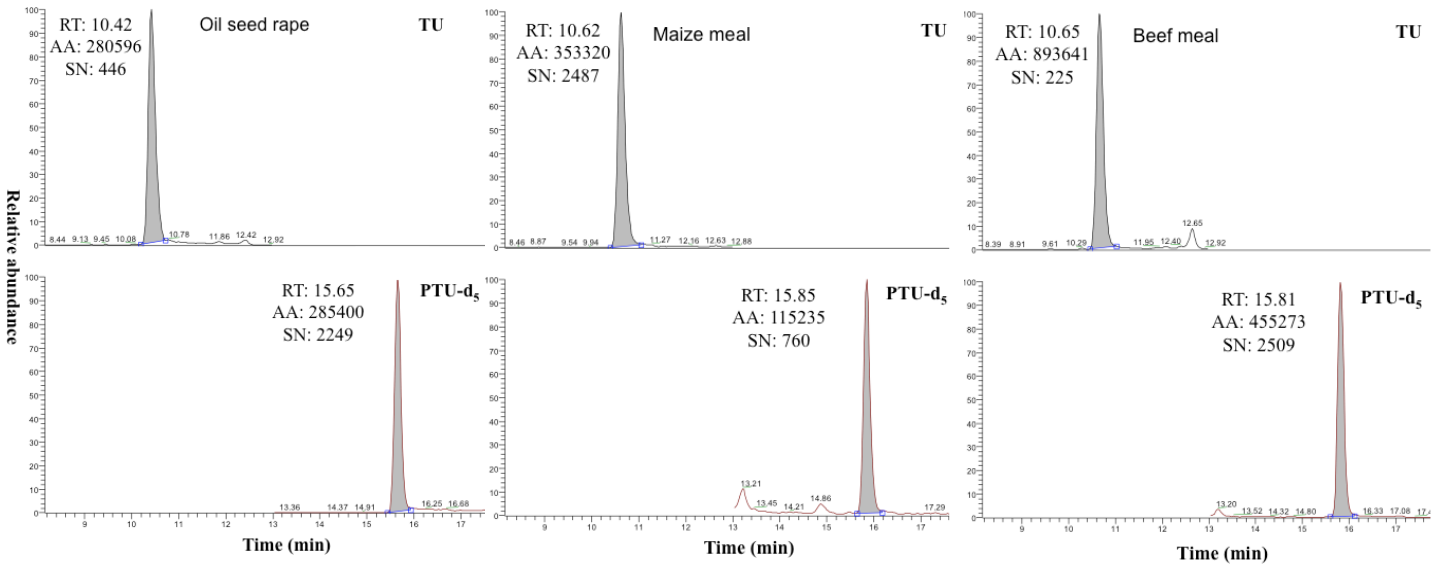


Figure IV.5: Chromatographic representation of detection of thiouracil and the internal standard PTU-d_5, in respectively: oil seed rape, maize meal, beef meal.

4. CONCLUSIONS

This study presents a new, successfully validated LC-MS^2 method for TU analysis from animal feeds, which differentiates itself by a statistically optimised extraction procedure adapted to both, Brassicaceae and non-Brassicaceae feedstuffs. The efficiency of the extraction and the purification steps, and the use of an adapted internal standard allowed obtaining a rugged method. The method was subjected to a careful validation according to common procedures i.e. CD 2002/257/EC guidelines [33]. In this validation accuracy, specificity, selectivity, repeatability, within-laboratory reproducibility, LOD, LOQ and linearity were tested demonstrating excellent performance and practical applicability in various animal feeds. Currently, the presence of natural and exogenous TU cannot yet be differentiated. Feed

analysis remains, therefore, a very important tool for indirect confirmation of natural TU occurrence. Moreover, the latest reflection paper by the EURL [61] also advised the analysis of animal feed when TU concentrations above > 30 µg L^{-1} are encountered in livestock urine in national control plans. It may be clear that this method for the analysis of TU in Brassicaceae and non-Brassicaceae feeds could easily suit that purpose.

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6. REFERENCES

[1] **Khera, K. S.** 1973. Ethylenethiourea - Teratogenicity Study in Rats and Rabbits. Teratology **7:**243-252.

[2] **Martinez-Frias, M. L., A. Cereijo, E. Rodriguezpinilla, and M. Urioste.** 1992. Methimazole in Animal Feed and Congenital Aplasia Cutis. Lancet **339:**742-743.

[3] **Eghbalian, F.** 2007. Aplasia Cutis Congenita after methimazole exposure in utero; a case report and literature review. Iranian Journal of Pediatrics **17:**293-296.

[4] **International Agency for Research on Cancer.** 2010. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Available online on: http://monographs.iarc.fr/ENG/Classification/index.php

[5] **European Community.** 1981. Council Directive 81/602/EC. Official Journal of the European Communities **L 222:**32-33.

[6] **Pinel G, Maume D, Deceuninck Y, André F, Le Bizec B.** 2006. Unambiguous identification of thiouracil residue in urine collected in non-treated bovine by tandem and high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry **20:**3183-3187.

[7] **Vanden Bussche, J., L. Vanhaecke, Y. Deceuninck, K. Wille, K. Bekaert, B. Le Bizec, and H. F. De Brabander.** 2011. Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry detection of naturally occuring thiouracil in urine of untreated livestock, domesticated animals and humans. Food Additives and Contaminants **28:**166-172.

[8] **European Food Safety Authority.** 2010. Report for 2008 on the results from the monitoring of veterinary medicinal product residues and other substances in food of animal origin in the Member States. European Food Safety Authority Journal **8**:1559. doi:10.2903/j.efsa.2010.1559.

[9] **Pinel G, Bichon E, Pouponneau K, Maume D, André F, Le Bizec B.** 2005. Multi-residue method for the determination of thyreostats in urine samples using liquid chromatography coupled to tandem mass spectrometry after derivatisation with 3-iodobenzylbromide. Journal Chromatography A **1085:**247-252.

[10] **Lõhmus M, Kallaste K, Le Bizec B.** 2009. Determination of thyreostats in urine and thyroid gland by ultra high performance liquid chromatography tandem mass spectrometry. Journal Chromatography A **1216:**8080-8089.

[11] **Vanden Bussche J, Vanhaecke L, Deceuninck Y, Verheyden K, Wille K, Bekaert K, Le Bizec B, De Brabander HF.** 2010. Development and validation of an ultra-high performance liquid chromatography tandem mass spectrometry method for quantifying thyreostats in urine without derivatisation. Journal Chromatography A **1217:**4285-429.

[12] **Pinel, G., S. Mathieu, N. Cesbron, D. Maume, H. F. De Brabander, F. Andre, and B. Le Bizec.** 2006. Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyrostats in meat production. Food Additives and Contaminants **23:**974-980.

[13] **European Union Reference Laboratories, E. U. R. L.** 2007. EURL guidance document. Available online on: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf.

[14] **Zhou, C. H., J. G. Tokuhisa, D. R. Bevan, and A. Esen.** 2012. Properties of beta-thioglucoside hydrolases (TGG1 and TGG2) from leaves of Arabidopsis thaliana. Plant Science **191:**82-92.

[15] **Vanden Bussche, J., J. A. L. Kiebooms, N. De Clercq, Y. Deceuninck, B. Le Bizec, H. F. De Brabander, and L. Vanhaecke.** 2011. Feed or Food responsible for the presence of low-level thiouracil in urine of livestock and humans? Journal of Agricultural and Food Chemistry **59:**5786-5792.

[16] **Krul, C., C. Humblot, C. Philippe, M. Vermeulen, M. van Nuenen, R. Havenaar, and S. Rabot.** 2002. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic in vitro large-intestinal model. Carcinogenesis **23:**1009-1016.

[17] **Cheng, D. L., K. Hashimoto, and Y. Uda.** 2004. In vitro digestion of sinigrin and glucotropaeolin by single strains of Bifidobacterium and identification of the digestive products. Food and Chemical Toxicology **42:**351-357.

[18] **Tripathi, M. K., and A. S. Mishra.** 2007. Glucosinolates in animal nutrition: A review. Animal Feed Science and Technology **132:**1-27.

[19] **Kiebooms, J. A. L., J. Vanden Bussche, L. Y. Hemeryck, V. Fievez, and L. Vanhaecke.** 2012. Intestinal microbiota contribute to the endogenous formation of thiouracil in livestock. Journal of Agricultural and Food Chemistry **60:**7769-7776.

[20] **Bones, A. M., and J. T. Rossiter.** 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. Physiologia Plantarum **97:**194-208.

[21] **Rosa, E. A. S., and A. S. Rodrigues.** 2001. Total and individual glucosinolate content in 11 broccoli cultivars grown in early and late seasons. Hortscience **36:**56-59.

[22] **Wallig, M. A., R. L. Belyea, and M. E. Tumbleson.** 2002. Effect of pelleting on glucosinolate content of crambe meal. Animal Feed Science and Technology **99:**205-214.

[23] **Van Doorn, H. E., G. C. van der Kruk, G. J. van Holst, N. C. M. E. Raaijmakers-Ruijs, E. Postma, B. Groeneweg, and W. H. F. Jongen.** 1998. The glucosinolates sinigrin and progoitrin are important determinants for taste preference and bitterness of brussels sprouts. Journal of the Science of Food and Agriculture **78:**30-38.
[24] **Mithen, R. F., M. Dekker, R. Verkerk, S. Rabot, and I. T. Johnson.** 2000. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. Journal of the Science of Food and Agriculture **80:**967-984.
[25] **Tanii, H., T. Takayasu, T. Higashi, S. Leng, and K. Saijoh.** 2004. Allylnitrile: generation from cruciferous vegetables and behavioral effects on mice of repeated exposure. Food and Chemical Toxicology **42:**453-458.
[26] **Schöne, F., K. Winnefeld, E. Kirchner, M. Grun, H. Ludke, and A. Hennig.** 1990. Copper and Iodine in Pig Diets with High Glucosinolate Rapeseed Meal .3. Treatment of Rapeseed Meal with Copper, and the Effect of Iodine Supplementation on Trace-Element Status and Some Related Blood (Serum) Parameters. Animal Feed Science and Technology **30:**143-154.
[27] **Burel, C., T. Boujard, A. M. Escaffre, S. J. Kaushik, G. Boeuf, K. A. Mol, S. Van der Geyten, and E. R. Kuhn.** 2000. Dietary low-glucosinolate rapeseed meal affects thyroid status and nutrient utilization in rainbow trout (Oncorhynchus mykiss). British Journal of Nutrition **83:**653-664.
[28] **Tripathi, M. K., I. S. Agrawal, S. D. Sharma, and D. P. Mishra.** 2001. Effect of untreated, HCl treated or copper and iodine supplemented high glucosinolate mustard (Brassica juncea) meal on nutrient utilization, liver enzymes, thyroid hormones and growth of calves. Animal Feed Science and Technology **92:**73-85.
[29] **Zang, X. P., H. Tanii, K. Kobayashi, T. Higashi, R. Oka, Y. Koshino, and K. Saijoh.** 1999. Behavioral abnormalities and apoptotic changes in neurons in mice brain following a single administration of allylnitrile. Archives of Toxicology **73:**22-32.
[30] **Ahlin, K. A., M. Emanuelson, and H. Wiktorsson.** 1994. Rapeseed Products from Double-Low Cultivars as Feed for Dairy-Cows - Effects of Long-Term Feeding on Thyroid-Function, Fertility and Animal Health. Acta Veterinaria Scandinavica **35:**37-53.
[31] **Zukalova, H., and J. Vasak.** 2002. The role and effects of glucosinolates of Brassica species - a review. Rostlinna Vyroba **48:**175-180.
[32] **European Community.** 2003. Commission Regulation (EC) No 1035/2003. Official Journal of the European Community **L 150:**24-25.
[33] **European Community.** 2002. Commissions Decision 2002/657/EC. Official Journal of the European Community **L 221:**8-36.
[34] **Box, G. E., and J. S. Hunter.** 1961. 2k-P Fractional Factorial Designs Part I. Technometrics **3:**311-351.
[35] **Yu, G. Y. F., E. J. Murby, and R. J. Wells.** 1997. Gas chromatographic determination of residues of thyreostatic drugs in bovine muscle tissue using combined resin mediated methylation and extraction. Journal of Chromatography B **703:**159-166.
[36] **Blanchflower, W. J., P. J. Hughes, A. Cannavan, M. A. McCoy, and D. G. Kennedy.** 1997. Determination of thyreostats in thyroid and urine using high-performance liquid chromatography atmospheric pressure chemical ionisation mass spectrometry. Analyst **122:**967-972.
[37] **Buick, R. K., C. Barry, I. M. Traynor, W. J. McCaughey, and C. T. Elliott.** 1998. Determination of thyreostat residues from bovine matrices using high-performance liquid chromatography. Journal of Chromatography B **720:**71-79.
[38] **Pensabene, J. W., S. J. Lehotay, and W. Fiddler.** 2001. Method for the analysis of thyreostats in meat tissue using gas chromatography with nitrogen phosphorus detection and tandem mass spectrometric confirmation. Journal of Chromatographic Science **39:**195-199.
[39] **Asea, P. E., J. D. MacNeil, and J. O. Boison.** 2006. An analytical method to screen for six thyreostatic drug residues in the thyroid gland and muscle tissues of food producing animals by liquid chromatography with ultraviolet absorption detection and liquid chromatography/mass spectrometry. Journal of Aoac International **89:**567-575.
[40] **Broekaert, N., C. Van Peteghem, E. Daeseleire, D. Sticker, and C. Van Poucke.** 2011. Development and validation of an UPLC-MS/MS method for the determination of ionophoric and synthetic coccidiostats in vegetables. Analytical and Bioanalytical Chemistry **401:**3335-3344.
[41] **Wrede, F**. 1941. Die Methoden der Fermentforschung, Georg Thieme, Leipzig, Bd2, 1835.
[42] **De Brabander, H. F., R. Verbeke.** 1982. Determination of oxazolidine-2-thiones in biological fluids in the ppb range. Journal of Chromatography A **252:**225-239.
[43] **Pollet, B., L. Vanhaecke, P. Dambre, P. Lootens, and K. Steppe.** 2011. Low night temperature acclimation of Phalaenopsis. Plant Cell Reports **30:**1125-1134.
[44] **Palmieri, S., O. Leoni, and R. Iori.** 1982. A Steady-State Kinetics Study of Myrosinase with Direct Ultraviolet Spectrophotometric Assay. Analytical Biochemistry **123:**320-324.
[45] **Shen, J. Z., S. X. Zhang, C. M. Wu, H. Y. Jiang, Z. H. Wang, and L. L. Cheng.** 2010. Determination of Six Resorcylic Acid Lactones in Feed by GC-MS. Chromatographia **71:**163-165.
[46] **Wang, W. Y., Y. L. Zhang, J. Y. Wang, X. Shi, and J. N. Ye.** 2010. Determination of beta-agonists in pig feed, pig urine and pig liver using capillary electrophoresis with electrochemical detection. Meat Science **85:**302-305.
[47] **Ying, Y. F., Y. L. Wu, Y. Wen, T. Yang, X. Q. Xu, and Y. Z. Wang.** 2013. Simultaneous determination of six resorcylic acid lactones in feed using liquid chromatography-tandem mass spectrometry and multi-walled carbon nanotubes as a dispersive solid phase extraction sorbent. Journal of Chromatography A **1307:**41-48.
[48] **Karnes, H. T., and C. March.** 1991. Calibration and Validation of Linearity in Chromatographic

Biopharmaceutical Analysis. Journal of Pharmaceutical and Biomedical Analysis **9:**911-918.
[49] **Youssef, Y. A., Y. Beauchamp, and M. Thomas.** 1994. Comparison of a Full Factorial Experiment to Fractional and Taguchi Designs in a Lathe Dry Turning Operation. Computers & Industrial Engineering **27:**59-62.
[50] **Plackett, R. L., J. P. Burman.** 1946. The design of optimum multifactorial experiments. Biometrika **33:**305-325.
[51] **Eriksson L, E. Johansson, N. Kettaneh-Wold, S. Wold.** 2008. Multi- and megavariate data analysis. Principles and applications, Umetrics Academy, Umea.
[52] **Beres, D. L., and D. M. Hawkins.** 2001. Plackett-Burman techniques for sensitivity analysis of many-parametered models. Ecological Modeling **141:**171-183.
[53] **Jia, J. S., H. B. Deng, J. Duan, and J. Z. Zhao.** 2009. Analysis of the major drivers of the ecological footprint using the STIRPAT model and the PLS method-A case study in Henan Province, China. Ecological Economics **68:**2818-2824.
[54] **Esteve-Romero, J., I. Escrig-Tena, E. F. Simo-Alfonso, and G. Ramis-Ramos.** 1999. Determination of thyreostatics in animal feed by micellar electrokinetic chromatography. Analyst **124:**125-128.
[55] **Perez-Ruiz, T., C. Martinez-Lozano, A. Sanz, and R. Galera.** 2005. An ultrasensitive method for the determination of thiouracil and phenylthiouracil using capillary zone electrophoresis and laser-induced fluorescence detection. Electrophoresis **26:**2384-2390.
[56] **Kong, D. X., Y. W. Chi, L. C. Chen, Y. Q. Dong, L. Zhang, and G. N. Chen.** 2009. Determination of thyreostatics in animal feeds by CE with electrochemical detector. Electrophoresis **30:**3489-3495.
[57] **Wozniak, B., S. Witek, I. Matraszek-Zuchowska, and J. Zmudzki.** 2014. Determination of the thyreostats in animal feeding stuffs using liquid chromatography-tandem mass spectrometry. Bulletin of the Veterinary Institute in Pulawy **58:**413-419.
[58] **Arioli, F., M. P. Gavinelli, M. L. Fracchiolla, A. Casati, M. Fidani, E. Ferrer, and G. Pompa.** 2008. Evaluation of boldenone formation and related steroids transformations in veal faeces by liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry **22:**217-223.
[59] **McCarrison, R**. 1933. The goitrogenic action of soybean and groundnut. Indian Journal of Medical Research **21:**179.
[60] **Velasco, P., P. Soengas, M. Vilar, M. E. Cartea, and M. del Rio.** 2008. Comparison of glucosinolate profiles in leaf and seed tissues of different Brassica napus crops. Journal of the American Society for Horticultural Science **133:**551-558.
[61] **European Union Reference Laboratories, E. U. R. L.** 2014. EURL reflection paper. Not yet available online.

CHAPTER V

Validated Ultra High Performance Liquid Chromatography-tandem Mass Spectrometry method for quantitative analysis of total and free thyroid hormones in bovine serum

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ABSTRACT

Thyroid hormones are essential hormones for regulating growth and development. Methods to accurately monitor low-levels (ppb-ppt) of these hormones in serum are needed to assess overall health, both from a clinical perspective as from environmental contaminant or drug exposures. In general, the separation of the free thyroid hormone fraction from animal sera is performed through labour intensive equilibrium dialysis, while detection of total and free thyroid hormone fractions in animals is done with commercially available radioimmunoassays (RIAs). This study reports newly developed analysis methods for both the total and free fractions of triiodothyronine (T3), reverse-triiodothyronine (rT3) and thyroxin (T4) from bovine serum, with a much higher specificity and selectivity than commercially available RIAs. The bovine serum extraction procedures of total and free T3, rT3, T4 were optimised with fractional factorial designs and consisted of, respectively, deproteinisation followed by liquid-liquid extraction, 30 kDA ultrafiltration and solid phase extraction. Both free and total thyroid hormone UHPLC-HESI-MS/MS based analysis methods were successfully validated. The limits of quantification for T4, rT3 and T3 amounted respectively 0.04 ng mL^{-1}, 0.05 ng mL^{-1}, 0.03 ng mL^{-1} for the total fraction, and 6.6 pg mL^{-1}, 2.6 pg mL^{-1} and 2.7 pg mL^{-1} for the free fraction. Individual recoveries of total and free thyroid hormone fractions ranged between 95.6-106.3% and 92.1-106.5%. Good results for repeatability and intra-laboratory reproducibility (RSD%) were observed, i.e. respectively ≤8.0% and ≤7.3% for the total and free fractions. Excellent linearity ($R^2 \geq 0.99$) and lack-of-fit was proven for both fractions. In conclusion, these methods show excellent in-house performance and possibilities for elaboration to application in other animal sera (e.g. feline, canine, equine).

Keywords: thyroid hormone, total, free, UHPLC-MS/MS, D-optimal, Plackett-Burman, bovine serum

1. INTRODUCTION

The thyroid gland governs the metabolism of all species through thyroid hormone secretion. Therefore, the evaluation of its status through analysis of thyroid hormones is of great value in a veterinary clinical setting but also in other research fields where an overall view of the thyroid status is required. More specifically in reference to contaminants, like endocrine disrupting chemicals (EDC's) (e.g.: organ chlorine pesticides and polybrominated diphenyl ethers)) [1-3], or to endogenous formation of certain residues affecting the thyroid function (e.g. thiouracil) [4,5] thyroid hormone analysis has proven its merits.

The thyroid gland is histologically characterised by large-follicular tissue with a monolayer of cells, which produce thyroxine (T4, 90%) and triiodothyronine (T3, 10%) [6]. Due to the highly vascularised gland tissue, T4 and T3 can be quickly released into the systemic circulation. Secretion of thyroid hormones is well regulated by a negative feedback mechanism involving, besides the thyroid gland, the hypothalamus and the pituitary gland [7,6]. Circulating T4 concentration is 50-60 times higher compared to T3, and both hormones are also partially bound to blood proteins, of which the thyroid-binding globulin (TGB) is the most relevant (70-80%) besides the other common plasma proteins, prealbumin and albumin, which bind the remaining fraction [7,6]. Consequently, only a very small non-bound free fraction of these hormones (0.03% of total T4 and 0.3% of total T3) remains available to directly access the target organs and this availability is known to be influenced by the physiologic status of the organism and may thus alter in case of disease [8,7]. Moreover, only T3 will exert any biologic activity since T4 needs an additional deiodination step in the peripheral tissues [7]. This clearly illustrates why the monitoring of free and total thyroid hormone concentrations, and the individual identification of both T3 and T4, is of major importance in a clinical setting.

A vast amount of publications on method development for the analysis of total and free thyroid hormones is available, but these mostly apply to human serum and focus solely on free or total hormone fractions [9,10], which have been reviewed elsewhere [11,12].

For human total and free thyroid hormone analysis the detection method of reference has been (radio)immunoassays (RIA/IA) [10-13]. But over the years, the evolutions in hyphenated mass spectrometry (MS) based techniques (gas chromatography (GC)-MS; liquid chromatography (LC)-MS and LC-MS/MS) have led to the increasing replacement of RIAs [14] in favour of more sensitive and specific mass spectrometric applications for human total and free thyroid hormone analysis

[15-18,10]. For total thyroid hormone analysis in particular, laborious sample preparations were common [19-21] with the first GC-MS applications, but the evolution towards LC-MS methods, with eventually high performance liquid chromatography isotope dilution tandem MS (HPLC-ID-MS/MS) applications, allowed faster and more generic analysis of total thyroid hormones in humans [22,23,9]. Free thyroid hormone extraction, on the other hand, is still generally based on equilibrium dialysis (ED) combined with immunoassays, but mass spectrometric applications have also been described, e.g. ED LC-ID-MS/MS, which has been put forward as a human reference method [16,24,12]. Nevertheless, ED has the disadvantage to be a labour intensive, imprecise, technically demanding and costly procedure, which is hard to perform in a clinical laboratory [25,11]. Therefore, ultrafiltration (UF) was proposed as a valuable alternative for ED [26,24,18], which further led to its implementation in many North American laboratories for human serum analysis nowadays [11]. The separation through UF takes in average about 30 min, whereas ED requires 17 h to 24 h [25,11]. Only very few publications deal with method development for thyroid analysis in non-human species [10] and none are able to both asses free and total fractions of thyroid hormones. In animal medicine, several immunoassays are commercially available that detect total thyroid hormones, while the free fraction is generally determined by an ED immunoassay combination (e.g.: in donkeys, dogs and cats) [27-29]. Since thyroid hormones are involved in a variety of critical metabolic pathways and growth processes, it is clear that animals, veterinarians and researchers would benefit from the development of a fast, cost-effective and specific method allowing thyroid hormone monitoring. This paper therefore describes the development and validation [30] of rapid and sensitive methods combining ultra high performance liquid chromatography (UHPLC) with MS/MS detection preceded by two fast and cost-effective extraction procedures, for free and total thyroid hormone fractions in bovine serum, respectively.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The analytes, 3,5,3',5'-tetraiodothyronine (T4); 3,3',5-triiodothyronine (T3); 3,3',5'-triiodothyronine (reverse T3; rT3) were purchased from Sigma-Aldrich (St. Louis, USA). The internal standard, 3,5,3',5'-tetraiodothyronine-$^{13}C_6$ (T4-$^{13}C_6$) was acquired from Toronto Research Chemicals (Toronto, CA). All used solvents and reagents were obtained from VWR International (Merck, Darmstadt, Germany). Solvents were of 'pro

analysis' grade when used for extraction purposes and of 'LC-MS Optima' grade when used for U-HPLC-MS/MS application. Ultrapure water (UPW) was obtained from a purified-water system (Sartorius Stedim Biotech, GmbH arium 611 UV, Germany).
Primary stock solutions were prepared in LC-MS optima grade methanol (MeOH) with 25% ammonium hydroxide (100 µL NH_4OH, 12 mL^{-1} MeOH) according to Van Houcke et al., [31] at a concentration of 200 µg mL^{-1} and stored in dark glass flasks at -20 °C. Working solutions were made up in MeOH, except for total thyroid hormone extraction where spiking solutions were made up in ethanol (EtOH) to match the deproteinisation conditions.

2.2. Sample preparation

2.2.1. Sample

Bovine blood (Belgian Blue) was collected from Belgian slaughterhouses (without anticoagulant) on the slaughter line during exsanguination. Subsequently, the coagulated blood was centrifuged for 20 min at 2,625x*g*. The obtained bovine serum was pooled and stored at -20 °C in falcon tubes.

2.2.2. Statistical designs for the extraction procedure

Statistical model designs were used to optimise the analytical extraction procedures of both, total and free, thyroid hormone fractions in bovine serum. The dependent variables that might significantly affect the extraction efficiency were screened with a Plackett-Burman fractional factorial design (PB) for the free thyroid hormone fraction and a D-optimal experimental design for the total thyroid hormone fraction. These variables were selected based on a literature survey for both free and total thyroid fractions [32,15,22,16,9,33,23,34,26,10,35,36]. This allowed further optimisation with only the influential variables through response surface modelling (RSM) (i.e. central composite face-centered design (CCF)). If a factor proved to be non-critical (p>0.05), the best level according to its effect, was chosen. Generally, this was the least time-consuming or most result effective level of the factor, which was then built into the protocol [37].
For free thyroid analysis, the 11 selected variables and their respective levels are shown in Table V.1. Based on this statistical design (PB), only 12 experimental runs and three central value runs were required generating enough information to calculate the main effect of each variable and to eventually specify a particular combination of variable levels. Subsequently, critical variables with a significant (p<0.05) influence

(conditioning, wash and elution volumes) were subjected to RSM optimisation, resulting in the final protocol.

Table V.1: Variables of the D-optimal (total thyroid hormone) and Plackett Burman (free thyroid hormone) designs for their respective extraction optimizations. (*: ACN= acetonitrile)

D-optimal design	**Variables**	**Level 1**	**Level 2**	**Level 3**	**Level 4**
Qualitative	Deproteinisation solvent	MeOH	Aceton	EtOH	ACN*
	Deproteinisation temperature	-20 °C	4 °C	20 °C	
	Clean-up/extraction	No clean-up	Oasis HLB SPE	LLE	
	Shake manner	vortex	shaking		
Quantitative	Serum volume	100 µL	1000 µL		
	Shake time	15 sec	60 sec		
	Deproteinisation time	10 min	30 min		
	Centrifugation time	5 min	15 min		
PB design	**Variables**	**Level 1**	**Level 2**		
Qualitative	Conditioning solvent	MeOH	ACN*		
	Equilibrating solvent	5% MeOH in UPW	UPW		
	Wash solvent	MeOH	ACN*		
	Elution solvent	ACN*	MeOH		
	SPE cartridge type	C18 EC	OASIS HLB		
Quantitative	Conditioning volume	2 mL	6 mL		
	Dilution volume (UPW)	3 mL	9.5 mL		
	Wash solvent (in UPW)	5 %	20 %		
	Wash volume	2 mL	6 mL		
	FA in elution solvent	0%	0.1%		
	Volume elution solvent	1 mL	3 mL		

For total thyroid analysis, eight variables were selected with up to four levels per variable, as shown in Table V.1. Therefore, a total of 26 experimental runs and three central value runs were needed to specify this particular combination of settings. After performing the prescribed scenario, the main effect of each variable was calculated. Subsequently, the critical variables (p<0.05) (serum volume) and the most potent sample preparation procedure (liquid-liquid extraction) (p>0.05) were subjected to further optimisation by response surface methodology (CCF design). First the number of liquid-liquid extraction steps was evaluated (1.5 mL-1 mL/ 3 mL-3 mL-2 mL/ 5 mL-4 mL/ 5 mL-4 mL-3 mL). Subsequently, the volumes in the first (1.5-3 mL) and second (1-3 mL) liquid-liquid extraction steps were optimised along with the serum volume (100-1000 µL) in a CCF setup.

The software program Modde 5.0 (Umetrics, Umea, Sweden) was used to build the experimental design matrix and carry out data analysis. The parameters were inserted

into the program and depending on the purpose of the analysis (screening or RSM), the most adequate model was proposed by the program. Evaluation of the models was done through a one-way variance analysis (ANOVA) test. Significance was proven when *p*-values were smaller than 0.05.

2.2.2.1. Free thyroid hormone extraction

After statistical evaluation, a final extraction procedure for free thyroid hormones (Figure V.1) was defined

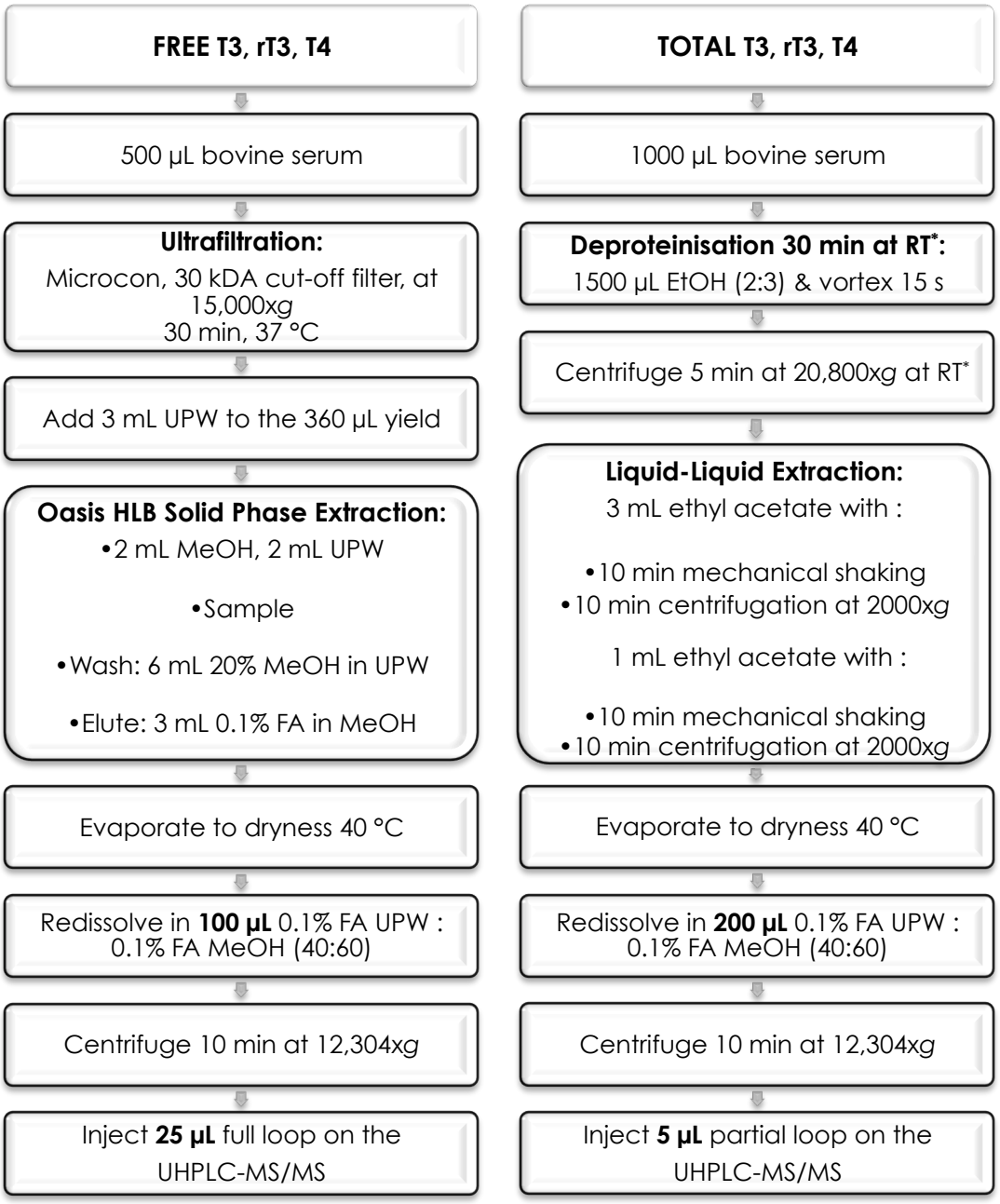

Figure V.1: Final extraction protocols after statistical optimization for free and total thyroid hormones in bovine serum. (*: RT=room temperature)

Briefly, 500 µL of thawed bovine serum was centrifuged using a UF centrifugal device (0.5 mL, 30 kDA, Amicon, Millipore, Belgium) at 37 °C approximating physiological conditions [16,11], separating the bounded hormone fraction from the free thyroid hormone fraction. The deproteinated serum was subsequently spiked with 100 pg mL^{-1} of internal standard ($^{13}C_6$-thyroxine) in MeOH. Next, samples were diluted with 3 mL UPW before performing solid phase extraction (SPE) with Oasis HLB cartridges (60 mg, 3cc, Waters GmbH, Germany). The eluted samples were evaporated to dryness and redissolved in 100 µL of the initial UHPLC conditions i.e. acidified (0.1% formic acid (FA)) UPW and 0.1% FA in MeOH (40:60), followed by centrifugation. Finally samples were conveyed into the insert of a glass amber vial for analysis.

2.2.2.2. Total thyroid hormone extraction

To establish the total thyroid hormone extraction from serum (Figure V.1), 1 mL of thawed bovine serum was deproteinated with a total amount of 1500 µL of EtOH (ratio 2:3) [9], which included the internal standard $^{13}C_6$-thyroxine (10 ng mL^{-1}). The deproteinated fraction was removed by a short high-speed centrifugation step (20,800x*g*, 5 min at room temperature) causing a plaque to form. Subsequently, liquid-liquid extraction (LLE) was performed on the supernatants. Next, the pooled LLE supernatants were evaporated to dryness and redissolved in 200 µL of the initial UHPLC conditions, followed by centrifugation. This mixture was conveyed into a glass amber vial with insert for analysis.

2.3. Instrumentation

2.3.1. Ultra High Performance Liquid Chromatography

Chromatographic separation of T4, T3 and rT3 was achieved by an Accela UHPLC system (Thermo Fisher Scientific, San José, USA), equipped with a reversed-phase C_{18} Hypersil gold column (100 × 2.1 mm, 1.9 µm, Thermo Fisher Scientific, San Jose, USA) at a column oven temperature of 35 °C. The optimised elution gradient consisted of acidified (0.1% FA) UPW (A) and acidified (0.1% FA) MeOH (B) at a flow rate of 0.3 mL min^{-1} over an 8-minute run. Starting conditions of 40%(A): 60%(B) were held up for 1.7 min, subsequently increasing to 37%(A): 63%(B) till 3.5 min, then slowly augmented to 35%(A): 65%(B) till 3.8 min, after which the gradient increased to a 100% MeOH (B) until 4.5 min. Between 4.5-5.5 min 100% MeOH was sustained, followed by a steep decrease to the initial conditions. Injection settings for total and free thyroid hormone analysis methods were respectively, partial loop (5 µL) and full loop (25 µL).

2.3.2. Triple-Stage Quadrupole Tandem Mass spectrometry

Mass spectrometric analysis was performed on a TSQ Vantage™ triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San José, USA) equipped with a second-generation heated electrospray ionisation (HESI-II™) interface in the positive ion mode using selected reaction monitoring (SRM). First MS-parameters (Table V.2), were optimised for each analyte separately by individually infusing the component's standard (10 ng μL^{-1} at 5 µL min^{-1}) and simultaneously the four most intense precursor ions were selected for each analyte. Subsequently, the remaining optimal instrumental parameter values were determined by evaluating signal intensity and signal-to-noise ratio of the precursor ions based on subsequent runs of a standard mixture under various parameter settings. Following instrumental parameters were found to be optimal: a cycle time of 1.2 s, a Q1 peak width of 0.70 Da and a collision gas pressure of 1.5 mTorr. Auxiliary, sheath and ion sweep gas were set at, respectively: 25, 45 and 2 au (arbitrary units). Vaporizer and capillary temperature of the HESI II probe were set at 395 °C and 330 °C. Finally, for the positive ion mode, the spray voltage was set at 4000 V. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific, San José, USA).

Table V.2: Precursor and product ion *m/z* values of the thyroid hormones and their internal standard along with the respective collision energy and S-lens value.

Precursor mass (*m/z*)		Product ions (*m/z*)	S-lens (V)	Collision energy (eV)
T3	*651.9*	198.1; 225.0; 478.9; 605.9	119	44; 44; 34; 21
rT3	*651.9*	478.9; 507.9; 605.9; 634.8	131	38; 22; 21; 16
T4	*777.8*	323.9; 350.9; 604.9; 731.8	144	45; 44; 40; 25
T4-$^{13}C_6$	*783.6*	329.8; 356.8; 639.8; 737.7	136	51; 43; 25; 20

2.3.3. Quality Assurance

Prior to the sample analysis, a standard mixture of the targeted compounds was injected to check the operational conditions of the UHPLC-MS/MS device. To every sample the internal standard T4-$^{13}C_6$ was added at a concentration of 10 ng mL^{-1} (total hormone) and 100 pg mL^{-1} (free hormone) prior to extraction. Identification of the thyroid hormones was based on their retention time relative to that of the internal standard and *m/z* ratios of selected product ions (ion ratios). After identification, the analyte's concentration was calculated by fitting its area ratio in a 10-point calibration curve. Area ratios were determined by integration of the area under the

specific selected reaction monitoring (SRM) chromatogram of an analyte in reference to the integrated area of the internal standard. The calibration curves were extracted in pooled bovine serum and fortified at the following concentrations: 7.5-15-30-60-90-120-180-360-540 ng mL^{-1} for T4, 0.25-0.5-1-2-3-4-6-12-18 ng mL^{-1} for rT3, 0.625-1.25-2.5-5-7.5-10-15-30-45 ng mL^{-1} for T3 for total thyroid hormone analysis. The free thyroid hormone calibration curves were spiked at the concentrations of 12.5-25-50-100-150-200-300-600-900 pg mL^{-1} for T4 and from 5-10-20-40-60-80-120-240-360 pg mL^{-1} for rT3 and T3.

2.4. Validation

Once the extraction procedure for both methods had been optimised, the total and free thyroid hormone analytical methods were validated in order to show their applicability and robustness. To this extent, a validation protocol in accordance with the CD 2002/657/EC [30] was adopted. Therefore, non-spiked bovine serum samples (n=20) were analysed to check the ruggedness of each method and to determine the endogenous levels of T4, T3 and rT3 for both the free and total thyroid hormone fraction. The bovine serum samples were subsequently fortified in order to determine the specificity of each peak based on retention time and signal amplitude increase according to the fortification level (n=3). This has been reported as a common approach when dealing with endogenous background levels [38-40]. For the validation of the total thyroid hormone fraction method, pooled bovine serum samples (6 per level) were fortified with 3 different concentrations (T4: 30-60-90 ng mL^{-1}, T3: 2.5-5-7.5 ng mL^{-1} and rT3: 1-2-3 ng mL^{-1}). For the validation of the free thyroid hormone fraction method, pooled bovine serum samples were fortified accordingly: T3 and rT3 at 20-40-60 pg mL^{-1} and T4 at 50-100-125 pg mL^{-1}. The quantification was based on matrix matched calibration curves consisting of 9 fortification levels. The linearity of the curves was evaluated through the coefficient of determination (R^2), which should be ≥ 0.99 [30] and the lack-of-fit (LOF), which is a well-known linearity evaluator [41]. The LOF was assessed for free and total thyroid hormones, taking into consideration the deviation of linearity of the datasets by building an univariate linear regression model (SPSS 20, IBM, USA) with the calibration curve replicates (n=3), setting concentration as independent variable and the respective area ratios (e.g. area compound/area respective internal standard) as dependent variable. Based on the analysis of blank samples, an average naturally occurring concentration could be deduced for each analyte. The obtained values were subtracted from the fortified samples enabling the calculation of the actual fortified free or total thyroid

hormone concentrations [42,43]. Thyroxine-$^{13}C_6$ was selected as an appropriate $^{13}C_6$-labelled internal standard [33,16,18,10].

2.5. Statistical analysis

Linearity evaluation was performed for both methods through lack-of-fit analysis (*F*-test) of the calibration curves in SPSS 20 (IBM, USA). All statistical evaluation in relation to the designs was performed with Modde 5.0 (Umea, Sweden).

3. RESULTS AND DISCUSSION

3.1. Statistical optimisation of free thyroid hormone analysis: Plackett-Burman

The strength of fractional designs (e.g. Plackett-Burman) is their ability to provide a significant time and effort reduction without losing valuable information, but still producing comparable results to the laborious full factorial design [44]. The PB design [45] is therefore a useful screening tool to distinguish from a pool of variables those variables that might affect the response of the component(s) of interest. Consisting of a two-level design (2^{k-1}), PB investigates k-1 variables, with k runs, where k is a multiple of four (e.g. 11 factors, 12 runs). In comparison, an x^k full factorial design requires all possible combinations to be tested of all (x) levels for each of (k) factors, which dramatically increases the amount of runs (e.g. 11 factors, 2024 trials) [46]. The amount of runs will generally be augmented with three center-points to obtain informative data about the range between the upper and lower limit of the variables [47].

In this study, the PB design was made up of 11 scientifically based factors (Table V.1), which enabled a first screening of the important variables in free thyroid hormone extraction [26,16,24,18]. This resulted in 5 significant factors ($p<0.05$) i.e. conditioning, washing and elution volume, as well as elution solvents (MeOH, acetonitrile (ACN)) (Figure V.2).

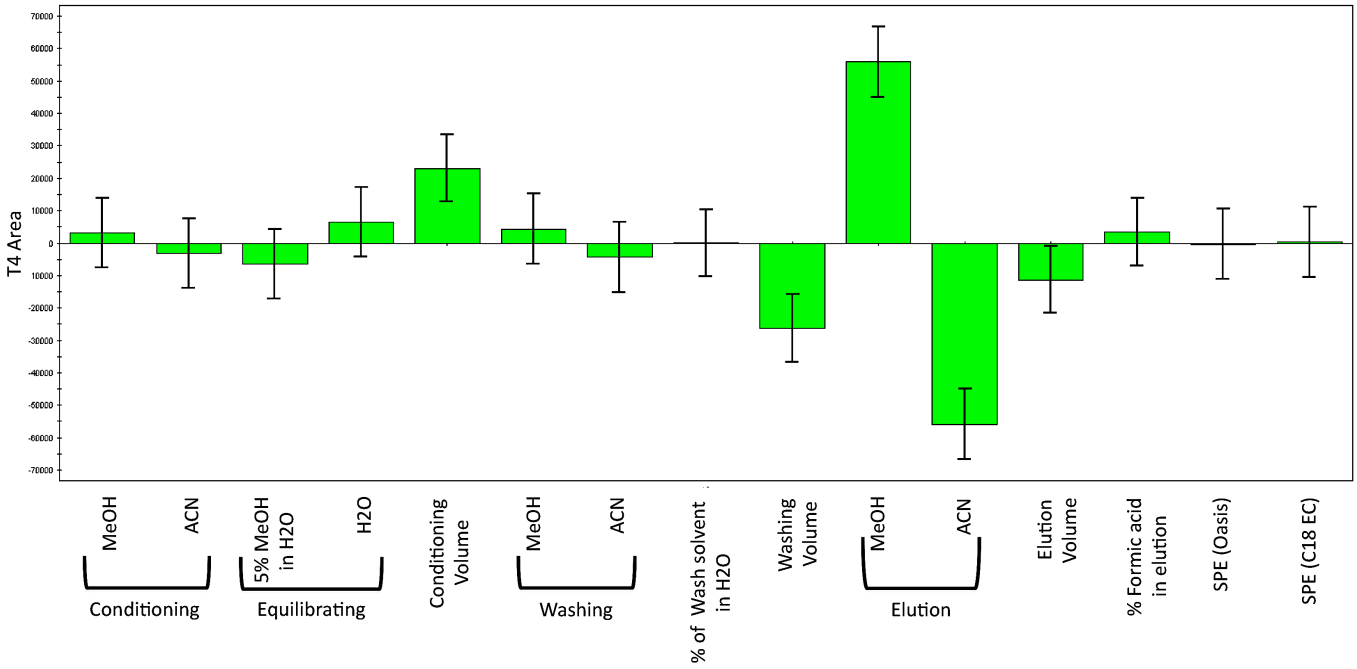

Figure V.2: The Plackett-Burman coefficients plot for free T4 with conditioning volume, washing volume, elution solvents (MeOH and ACN) and elution volume, as significant factors (p<0.05).

The type of SPE cartridge did not significantly enhance extraction (p>0.05), therefore the Oasis HLB (3cc, 60 mg, Waters) was preferred over the C18 (EC) cartridge based on literature [33], as well as, an overall slight non-significant positive effect. Next, a sensitivity analysis of the critical PB factors was implemented by means of a CCF full factorial design to gain insight in the possible interactions between factors [48,37]. Typically in a CCF, all scenarios per factor (conditioning, washing and elution volume) are evaluated at low, high but also at midlevel ranges. The latters are called star-points and are set at the center of each face of the factorial space and chosen within the range of each factor [47]. Evidently, because the number of scenarios becomes equal to 2^k, including a lot of parameters (k) becomes less feasible. Hence, a screening model was applied to the variables in this study, so the remaining variables were limited in number for further optimisation in the full factorial model with response surface modelling (CCF). Finally, based on the evaluation of the CCF, the solid phase extraction parameters (conditioning (2-6 mL), wash (2-6 mL) and elution volume (1-3 mL)) could be optimised with respect to their effects (p>0.05) in response surface plots.

3.2. Statistical optimisation of the total thyroid hormone analysis: D-optimal design

D-optimal designs are based on a mathematical formulation of the expected relations between variables [49], which mostly own, but do not require, an orthogonal matrix design resulting in possible correlated effect estimates. They are very useful when classical symmetrical designs (fractional or factorial) do not apply, e.g. the number of experiments chosen by a classical design is too large (e.g.: qualitative factors have more than two levels, which was the case here), when the experimental region is not regular in shape [50]. Moreover, the results of the experimental design can immediately be interpreted and show the effects of individual factors, indicating the optimal conditions, including their robustness [49]. For total thyroid hormones a D-optimal design was chosen since various multi-level qualitative factors were included and the amount of runs was lower than a fractional factorial design would have implied [51,47].

The D-optimal optimisation based on nine variables (Table V.2) resulted in the elucidation of three significant factors ($p<0.05$): serum volume, mechanical vortex and manual shaking (Figure V.3).

Significance could not be proven for the remaining factors, since these had merely non-critical (positive/negative) effects ($p>0.05$) on the extraction procedure. When the observed effect of a variable, which either aimed towards the upper or lower level of the chosen range, was similar for the majority of the components this level was built in. Since liquid-liquid extraction (LLE) had been previously described in literature as a potential extraction procedure for total thyroid hormones [33] and, since the effect of SPE ($p>0.05$) was not more advantageous, LLE was included in the RSM using a CCF design along with the serum volume (500-1000 µL). The evaluation of the number of liquid-liquid extraction steps resulted in a significant positive effect ($p<0.05$) for the two-step 1.5 mL-1 mL extractions. Subsequently, the optimization of the volumes in the first (1.5 mL-3 mL) and second (1 mL-3 mL) liquid-liquid extraction steps along with the serum volume (100-1000 µL) showed a positive effect ($p>0.05$) for the serum volume and an overall positive effect for the higher volume in the first liquid-liquid extraction step. Therefore, the most promising settings were built into the extraction procedure resulting in a serum volume of 1000 µL and a 3-1 mL liquid-liquid extraction.

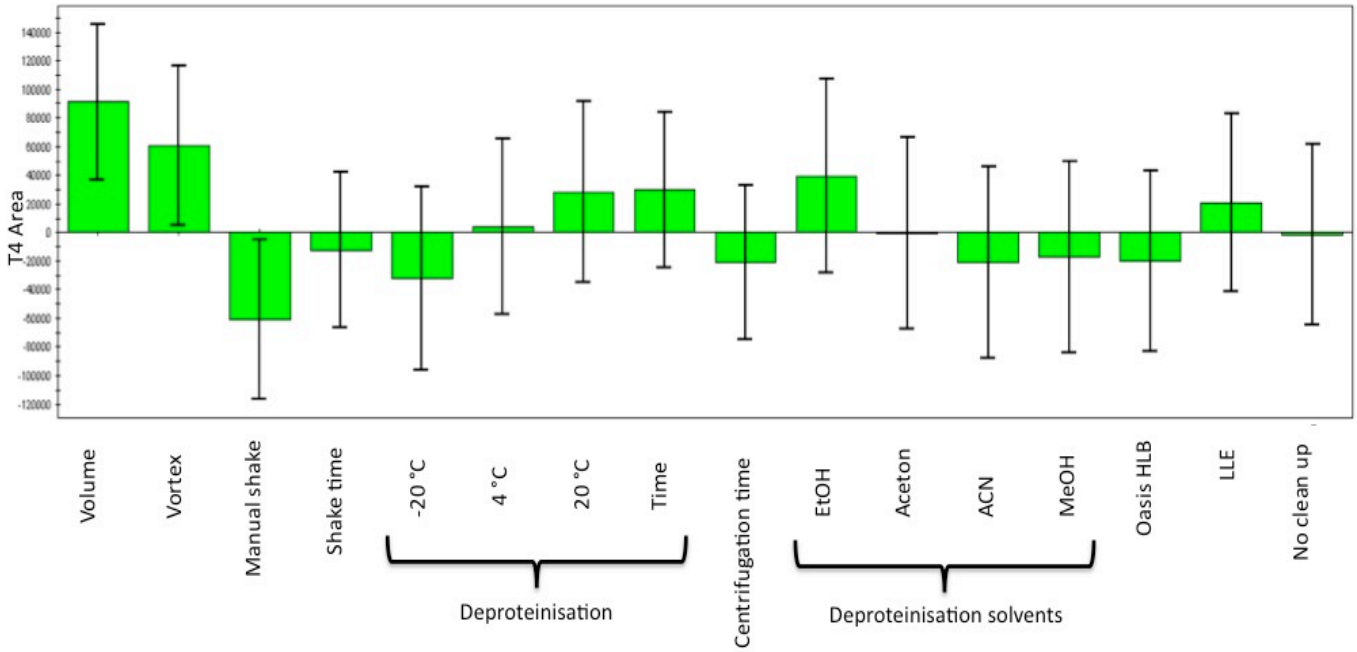

Figure V.3: The D-optimal coefficients plot for free T4 with conditioning volume, mechanical vortex and manual shake, as significant factors (p<0.05).

3.3. Optimisation of UHPLC and MS-parameters

For the elaboration of the UHPLC method, various parameters were optimised to overcome the lack of mass difference between T3 and rT3 (m/z 651.9) and subsequent similarity of their product ions (m/z 478.9-605.9), which called for their separation in time. Therefore, a variety of UHPLC-columns were tested under various flow (250-500 µL min^{-1}) conditions: Hypersil Gold (50-100 x 2.1 mm, 1.9 µm, Thermo Scientific), Acquity HSS T3 (50 x 2.1 mm, 1.8 µm, Waters), C_{18} Sphinx RP (50 x 2.0 mm, 1.8 µm, Machery-Nagel), Nucleodur C_{18} Gravity EC and Nucleodur C_{18} Isis EC (50-100 x 2.0 mm, 1.8 µm, Machery-Nagel). Based on the signal-to-noise ratio, peak area, peak shape (asymmetry factor: 0.9-1.2) and baseline peak separation (above 1.5) between T3 and rT3, the Hypersil Gold (100 x 2.1 mm, 1.9 µm) column was retained. Two solvent combinations were tested: acidified (0.1% FA) UPW/acidified (0.1% FA) MeOH [15,18,20] and acidified (0.2% FA) UPW/acidified (0.2% FA) 1:1 mixture of MeOH:ACN [35]. Additional separation was achieved by careful optimisation of the gradient program. Therefore, various gradient programs with 0.1% aqueous FA and 0.1% FA in MeOH were tested, as this was the most optimal solvent combination in terms of signal-to-noise ratio. The gradient program was extended to an 8 min run since this considerably reduced matrix interferences [11] and soiling of the column and MS over a large batch of samples.

Before determining the optimal MS conditions, all 3 thyroid hormones and the $^{13}C_6$-labelled internal standard were infused (10 ng μL^{-1}) into the tandem MS/MS and *m/z* values of the precursor ion, product ions and their respective collision energies and S-lens voltages were defined (Table V.2). For every analyte (precursor ion), four daughter ions (product ions) with the highest signal intensity and signal-to-noise ratios were selected. Subsequently, optimal instrumental parameter values were determined by evaluating signal intensity and signal-to-noise ratio of selected precursor ion based on subsequent runs of a standard mixture of all four components. Identification of the thyroid hormones found in the bovine serum was therefore based on both retention time and the *m/z* ratio of four precursor ions. As such, the application of UHPLC-HESI-MS/MS with SRM allowed the detection of total and free T4, T3 and rT3 without significant interferences from the serum (cfr. *2.4.1 Specificity and selectivity*).

3.4. Method validation for free and total thyroid hormones

The analytical methods were validated according to the criteria specified in CD 2002/657/EC for quantitative confirmation [30]. An appropriate $^{13}C_6$-labelled internal standard, T4-$^{13}C_6$, was chosen for its capability to anticipate fluctuations in the signal intensity upon extraction [16,10]. Indeed, the physico-chemical similarity between ^{2}H- or ^{13}C-labelled internal standards and the analyte of interest is known to be greater for ^{13}C-labelled analogues. Moreover, the use of ^{13}C-labelled internal standards is preferred for its improved ion suppression compensating ability, which is important in MS/MS [52].

Spiked thyroid hormone concentrations were based on background levels in the analysed bovine sera and reported concentrations. For the total thyroid hormone fraction, concentrations have been reported in the range of 36.0-73.0 ng mL^{-1} for T4, 0.6-1.6 ng mL^{-1} for T3 and 0.2-0.3 ng mL^{-1} for rT3 in bovine serum. For the free thyroid hormone fraction, concentrations of 3.0-6.2 pg mL^{-1} for T3 and 10-18.6 pg mL^{-1} for T4 have been reported in bovine serum [53,54]. All these reported concentrations [53-58] are of course highly dependent of the physiological status of the animal (e.g. lactation, illness, age, bread) and thus not restrictive in any way.

3.4.1. Specificity and selectivity

Bovine serum contains a certain background level of T4, T3 and rT3 as these are naturally occuring hormones. The chromatograms of 20 blanks indicated that no

other matrix substances significantly interfered with the thyroid hormone signals, since signal-to-noise ratios of the chromatograhic peaks were in all cases higher than 10 (Figures V.4 and V.5). When samples were fortified, a significant increase of the peaks of interest at the expected retention times with a substantial augmentation in area and signal-to-noise ratio were observed, thereby proving its selectivity for the analytes of interest. In accordance with CD 2002/657/EC, analytes were identified on the basis of their relative retention time, i.e. the ratio of the retention time of the analyte to that of the internal standard [30]. As a result, the developed method was found to be specific and selective for T4, T3, rT3 in the presence of matrix compounds.

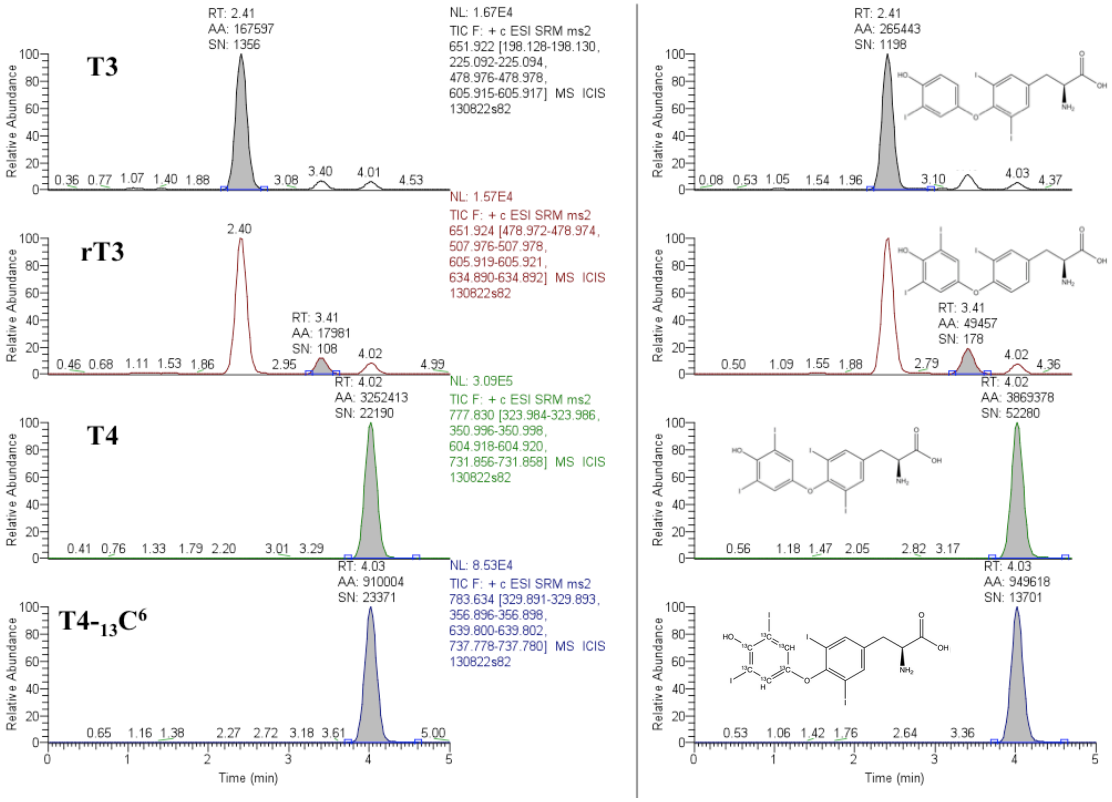

Figure V.4: UHPLC-MS/MS total ion chromatograms of total T3, rT3, T4 in (left) blanc and (right) spiked (0.625; 0.250; 7.5 ng mL^{-1}) bovine serum with added internal standard T4-$^{13}C_6$ (10 ng mL^{-1}) and respective retention time (RT), area (AA) and signal-to-noise ratio (SN).

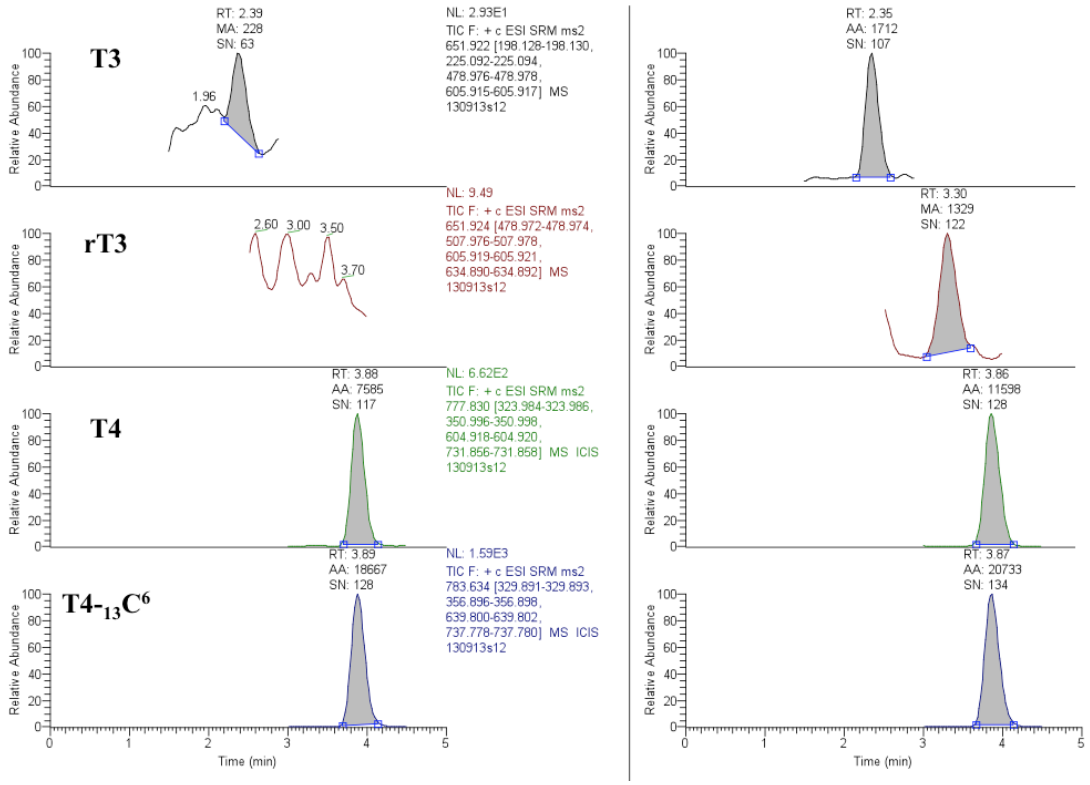

Figure V.5: UHPLC-MS/MS total ion chromatograms of free T3, rT3, T4 in (left) blank and in (right) spiked (10; 10; 25 pg mL^{-1}) bovine serum with added internal standard T4-$^{13}C_6$ (100 pg mL^{-1}) and respective retention time (RT), area (AA) and signal-to-noise ratio (SN).

3.4.2. Linearity

A 10-point calibration curve in pooled bovine serum was used to evaluate the linearity of the developed method for the different compounds in both extraction procedures. The samples were fortified with concentrations reported under 1.3.3 *Quality assurance*. For each sample the endogenous concentration, which was calculated as the average concentration based on the 20 non-spiked samples, was subtracted from the calculated total concentration. Linearity performed well since determination coefficients (R^2) were all ≥ 0.99, which complies with similar reports [24,10], as well as, the EC/2002/657 directive requirements [30]. Furthermore, the regression model equations resulting from lack-of-fit [41] were reported to be all linear (*F*-test; α=0.05).

3.4.3. Precision

The precision of the developed analytical method was evaluated through repeatability and within-laboratory reproducibility determination. Both validation parameters were evaluated by calculating their relative standard deviations (RSD%). To assess the repeatability, three series of six replicates at three different fortification levels in bovine serum were analysed by the same operator under repeatable conditions (Table V.3 and V.4).

Table V.3: Performance characteristics of the total (T) thyroid hormone UHPLC-MS/MS method from bovine serum (ng mL^{-1}).

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Nominal concentration (ng mL^{-1})	Recovery (%)	Precision	
					Repeatability RSD (%)	Intra-lab. reprod. RSD (%)
T T4	0.01	0.04	30	104.4	6.0	5.2
			60	106.3	5.4	5.0
			90	97.1	5.7	4.7
T T3	0.01	0.03	2.5	103.9	4.8	4.4
			5	100.5	3.9	3.9
			7.5	95.6	4.1	3.7
T rT3	0.02	0.05	1	102.7	6.2	5.4
			2	97.2	5.3	4.9
			3	98.1	2.5	2.5

Table V.4: Performance characteristics of the free (f) thyroid hormone UHPLC-MS/MS method from bovine serum (pg mL^{-1}).

Analyte	LOD (pg mL^{-1})	LOQ (pg mL^{-1})	Nominal concentration (pg mL^{-1})	Recovery (%)	Precision	
					Repeatability RSD (%)	Intra-lab. reprod. RSD (%)
f T4	2.2	6.6	50	98.3	6.9	7.1
			100	99.8	6.3	6.0
			125	101.8	7.5	6.3
f T3	0.9	2.7	20	92.4	4.2	4.4
			40	106.5	5.3	4.8
			60	102.7	6.7	6.2
f rT3	0.9	2.6	20	92.1	8.0	7.1
			40	105.6	6.7	6.2
			60	98.7	7.7	7.3

The calculated RSD% values were excellent for the total (2.5-6.2%) and free (4.2-8.0%) thyroid hormone fraction since values were well below 15% compliant with CD 2002/657/EC [30]. The intra-laboratory reproducibility was evaluated with four series of six replicates of samples, at three fortification levels analysed by different operators on different days. The calculated intra-laboratory RSD% values for free and total thyroid hormones ranged respectively between 4.4-7.3% and 2.5-5.4% (Table V.3 and V.4) for the three components over the various levels, which indicates a very good within-laboratory reproducibility, with reference to the 20% upper limit set by the CD 2002/657/EC [30]. Also, in comparison to scientific literature intra-laboratory and repeatability RSDs are found to be within range for both total and free hormone. For example, HPLC-ID-MS/MS methods have been reported with RSDs ranging from 4.1-9.0% for within-day and from 6.7-9.0% for between-day precision for free T4 and T3 in human serum [26,24]. For total T3 and T4 RSDs, within-day precision between 1.4-3.2% and between-day precision between 3.6-9.0% for total T3 and T4 have been reported in human serum [9]. Wang & Stapleton (2010) [10], which also worked with bovine serum, had intraday RSDs ranging from 1.2 to 9.6% for total T3, rT3 and T4, which closely correlate with our ranges.

3.4.4. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) are the lowest levels at which a compound can be identified or quantified with a signal-to-noise ratio (S/N) of respectively, ≥3 and ≥10. For the thyroid hormones (total and free) the detection and quantification limits were theoretically calculated based on 10-point calibration curves in matrix, correlating concentrations to signal-to-noise values.

For total and free thyroid hormone analysis our LOD and LOQ values were remarkably better compared to literature (Table V.3 and V.4) [22,9,26]. For free (f) T3 and T4 LODs in human serum of, respectively, 0.4 pg mL^{-1} [24] and 2.5 pg mL^{-1} [26,24] (HPLC-ID-MS/MS) have been described, which is in line with our results of 0.9-2.2 pg mL^{-1} with a UHPLC-MS/MS application. For total (T) thyroid hormones in human serum, LODs of 0.03 ng mL^{-1} for TT4 (LC-ID-ESI-MS) and of 0.062 ng mL^{-1} for TT3 and TT4 (LC-ID-MS/MS) have been attained when deproteinisation, with or without LLE and SPE, was applied [22,9]. Reported LOQs for total thyroid hormones amount at 0.15 ng mL^{-1} with LC-ID-MS/MS for TT3 and TT4 in human serum after deproteinisation [9], while an LOQ of 0.5 ng g^{-1} in lyophilised human serum for TT3 and TT4 were achieved, if the analytical technique was preceded by deproteinisation and SPE [33]. To the best of our knowledge the only UHPLC-MS/MS method reported in bovine serum, acquired LODs in the range of 0.3-1.4 ng mL^{-1} for five thyroid hormones [10]. Clearly, the LODs and

LOQs achieved in our study in bovine serum are well below the average reported values for total and free thyroid hormone analysis bearing in mind a simple liquid-liquid extraction and UF-SPE combination sufficed to achieve this with a similar tandem MS technique.

3.4.5. Mean recovery

As no certified reference material was available, trueness was determined as the mean corrected recovery by using fortified bovine serum samples. To this extent, three fortification levels were considered with six replicates for each level for both total and free fractions (Table V.3 and V.4). For each sample, background concentrations were deducted from the calculated concentrations when necessary. Results were found satisfactory for both total and free thyroid hormone methods, since the lowest mass fraction (10-100 ppb) according to the European directive 2002/657/EC [30] requires mean recoveries between 80-120% and the present recoveries ranged for free and total thyroid hormones fractions between 92.1-106.5% and between 95.6-106.3%, respectively. Reportedly, total and free thyroid hormone recoveries for T3 and T4 in human sera have been described between 90-109% with (HP)LC-ID-MS/MS detection [9,24]. In comparison with the only so far developed LC-MS/MS application in bovine serum [10] (81.3-111.9%) for total thyroid hormones, our newly developed method clearly performed better.

4. CONCLUSION

In this study, tailored extraction procedures were successfully developed for total and free T3, rT3 and T4 fractions, based on statistical fractional factorial designs in bovine serum. Both for free and total hormone fractions, one UHPLC-MS/MS detection method was developed and optimised operating in both the ng mL^{-1}, as well as, low pg mL^{-1} ranges. This resulted in the successful validation of both methods according to CD 2002/657/EC criteria, showing a good performance in terms of linearity, accuracy, precision, LOD and LOQ. Both free and total thyroid hormone methods have the potential to be broadened to a wider use (e.g. horse, dog, cat serum) in accordance to Wang & Stapleton (2010) [10], who developed a method for total thyroid hormone on bovine serum and applied it to human serum with nice correlations. In this context, it must be stressed that our method allows the analysis of both fractions, which to the best of our knowledge has not been reported earlier.

Therefore, in a veterinary clinical setting our newly developed sensitive and specific UHPLC-MS/MS technique would allow a fast and low-labour-intensive approach to quantify both thyroid hormone fractions (free and total) in non-human serum (bovine) and provide a sensitive and fast alternative in comparison with available methods.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

[1] **Rhind, S. M.** 2002. Endocrine disrupting compounds and farm animals: their properties, actions and routes of exposure. Domestic Animal Endocrinology **23:**179-187.

[2] **Rhind, S. M.** 2005. Are endocrine disrupting compounds a threat to farm animal health, welfare and productivity? Reproduction in Domestic Animals **40:**282-290.

[3] **Zoeller, T. R.** 2010. Environmental chemicals targeting thyroid. Hormones-International Journal of Endocrinology and Metabolism **9:**28-40.

[4] **Mandiki, S. N. M., N. Mabon, G. Derycke, J. L. Bister, J. P. Wathelet, R. Paquay, and M. Marlier.** 1999. Chemical changes and influences of rapeseed antinutritional factors on lamb physiology and performance - 2. Plasma substances and activity of the thyroid. Animal Feed Science and Technology **81:**93-103.

[5] **Kiebooms, J. A. L., J. Vanden Bussche, L. Y. Hemeryck, V. Fievez, and L. Vanhaecke.** 2012. intestinal microbiota contribute to the endogenous formation of thiouracil in livestock. Journal of Agricultural and Food Chemistry **60:**7769-7776.

[6] **Sjaastad, Ø. V., K. Hove, O. Sand,** The endocrine system, In: Physiology of the Domestic Animals, 2nd ed., Scandinavian Veterinary Press, Oslo, 2004, pp 200–232.

[7] **Yen, P. M.** 2001. Physiological and molecular basis of thyroid hormone action. Physiological Reviews **81:**1097-1142.

[8] **Schussler, G. C.** 2000. The thyroxine-binding proteins. Thyroid **10:**141-149.

[9] **Soukhova, N., O. P. Soldin, and S. J. Soldin.** 2004. Isotope dilution tandem mass spectrometric method for T4/T3. Clinica Chimica Acta **343:**185-190.

[10] **Wang, D. L., and H. M. Stapleton.** 2010. Analysis of thyroid hormones in serum by liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry **397:**1831-1839.

[11] **Soldin, O. P., and S. J. Soldin.** 2011. Thyroid hormone testing by tandem mass spectrometry. Clinical Biochemistry **44:**89-94.

[12] **Thienpont, L. M., K. Van Uytfanghe, K. Poppe, and B. Velkeniers.** 2013. Determination of free thyroid hormones. Best Practice & Research Clinical Endocrinology & Metabolism **27:**689-700.

[13] **Wu, A. H. B. and D. French.** 2013. Implementation of liquid chromatography/mass spectrometry into the clinical laboratory. Clinica Chimica Acta **420:**4-10.

[14] **Hoofnagle, A. N. and M. H. Wener.** 2009. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. Journal of Immunological Methods **347:**3-11.

[15] **De Brabandere, V. I., P. Hou, D. Stockl, L. M. Thienpont, and A. P. De Leenheer.** 1998. Isotope dilution-liquid chromatography electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method. Rapid Communications in Mass Spectrometry **12:**1099-1103.

[16] **Holm, S. S., S. H. Hansen, J. Faber, and P. Staun-Olsen.** 2004. Reference methods for the measurement of free thyroid hormones in blood evaluation of potential reference methods for free thyroxine. Clinical Biochemistry **37:**85-93.

[17] **Van Uytfanghe, K., D. Stockl, H. A. Ross, and L. M. Thienpont.** 2006. Use of frozen sera for FT4 standardization: Investigation by equilibrium dialysis combined with isotope dilution-mass spectrometry and immunoassay. Clinical Chemistry **52:**1817-1820.

[18] **Yue, B. F., A. L. Rockwood, T. Sandrock, S. L. La'ulu, M. M. Kushnir, and A. W. Meikle.** 2008. Free thyroid hormones in serum by direct equilibrium dialysis and online solid-phase extraction-liquid chromatography/tandem mass spectrometry. Clinical Chemistry **54:**642-651.

[19] **De Brabandere, V. I., D. Stockl, L. M. Thienpont, and A. P. De Leenheer.** 1998. On the use of trimethylchlorosilane in methanol for methylation of thyroxine prior to perfluoroacylation and isotope dilution gas chromatography mass spectrometry. Journal of Mass Spectrometry **33:**1032-1032.

[20] **Thienpont, L. M., C. Fierens, A. P. De Leenheer, and L. Przywara.** 1999. Isotope dilution-gas chromatography/mass spectrometry and liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of triiodo-L-thyronine in serum. Rapid Communications in Mass Spectrometry **13:**1924-1931.

[21] **Hantson, A. L., M. De Meyer, and N. Guerit.** 2004. Simultaneous determination of endogenous and C-13-labelled thyroid hormones in plasma by stable isotope dilution mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **807:**185-192.

[22] **Tai, S. S. C., L. T. Sniegoski, and M. J. Welch.** 2002. Candidate reference method for total thyroxine in human serum: Use of isotope-dilution liquid chromatography-mass spectrometry with electrospray ionization. Clinical Chemistry **48:**637-642.

[23] **Tai, S. S. C., D. M. Bunk, E. V. White, and M. J. Welch.** 2004. Development and evaluation of a reference measurement procedure for the determination of total 3,3 ',5-trilodothyronine in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Analytical Chemistry **76:**5092-5096.

[24] **Gu, J., O. P. Soldin, and S. J. Soldin.** 2007. Simultaneous quantification of free triiodothyronine and free thyroxine by isotope dilution tandem mass spectrometry. Clinical Biochemistry **40:**1386-1391.

[25] **Jonklaas, J., N. Kahric-Janicic, O. P. Soldin, and S. J. Soldin.** 2009. Correlations of Free Thyroid Hormones Measured by Tandem Mass Spectrometry and Immunoassay with Thyroid-Stimulating

Hormone across 4 Patient Populations. Clinical Chemistry **55:**1380-1388.
[26] **Soldin, S. J., N. Soukhova, N. Janicic, J. Jonklaas, and O. P. Soldin.** 2005. The measurement of free thyroxine by isotope dilution tandem mass spectrometry. Clinica Chimica Acta **358:**113-118.
[27] **Schachter, S., R. W. Nelson, C. Scott-Moncrieff, D. C. Ferguson, T. Montgomery, E. C. Feldman, L. Neal, and P. H. Kass.** 2004. Comparison of serum-free thyroxine concentrations determined by standard equilibrium dialysis, modified equilibrium dialysis, and 5 radioimmunoassays in dogs. Journal of Veterinary Internal Medicine **18:**259-264.
[28] **Wakeling, J., K. Moore, J. Elliott, and H. Syme.** 2008. Diagnosis of hyperthyroidism in cats with mild chronic kidney disease. Journal of Small Animal Practice **49:**287-294.
[29] **Fazio, E., P. Medica, C. Cravana, and A. Ferlazzo.** 2012. Total and free iodothyronines profile in the donkey (Equus asinus) over a 12-month period. Acta Veterinaria Brno **81:**239-244.
[30] **European Community.** 2002. Commissions Decision 2002/657/EC. Official Journal of the European Community **L 221:**8-36.
[31] **Van Houcke, S. K., K. Van Uytfanghe, E. Shimizu, W. Tani, M. Umemoto, and L. M. Thienpont.** 2011. IFCC international conventional reference procedure for the measurement of free thyroxine in serum. Clinical Chemistry and Laboratory Medicine **49:**1275-1281
[32] **Shihabi, Z. K.** 1993. Sample Matrix Effects in Capillary Electrophoresis .2. Acetonitrile Deproteinization. Journal of Chromatography A **652:**471-475.
[33] **Hopley, C. J., P. Stokes, K. S. Webb, and M. Baynham.** 2004. The analysis of thyroxine in human serum by an 'exact matching' isotope dilution method with liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry **18:**1033-1038.
[34] **Gika, H. G., V. F. Samanidou, and I. N. Papadoyannis.** 2005. Development of a validated HPLC method for the determination of iodotyrosines and iodothyronines in pharmaceuticals and biological samples using solid phase extraction. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **814:**163-172.
[35] **Svanfelt, J., J. Eriksson, and L. Kronberg.** 2010. Analysis of thyroid hormones in raw and treated waste water. Journal of Chromatography A **1217:**6469-6474.
[36] **Kunisue, T., J. W. Fisher, and K. Kannan.** 2011. Determination of Six Thyroid Hormones in the Brain and Thyroid Gland Using Isotope-Dilution Liquid Chromatography/Tandem Mass Spectrometry. Analytical Chemistry **83:**417-424.
[37] **Broekaert, N., C. Van Peteghem, E. Daeseleire, D. Sticker, and C. Van Poucke.** 2011. Development and validation of an UPLC-MS/MS method for the determination of ionophoric and synthetic coccidiostats in vegetables. Analytical and Bioanalytical Chemistry **401:**3335-3344.
[38] **Bean, K. A. and J. D. Henion.** 1997. Direct determination of anabolic steroid conjugates in human urine by combined high-performance liquid chromatography and tandem mass spectrometry. Journal of Chromatography B **690:**65-75.
[39] **Bekaert, K. M., J. Vanden Bussche, S. Francois, F. A. M. Tuyttens, H. F. De Brabander, F. Vandendriessche, and L. Vanhaecke.** 2012. A validated ultra-high performance liquid chromatography coupled to high resolution mass spectrometry analysis for the simultaneous quantification of the three known boar taint compounds. Journal of Chromatography A **1239:**49-55.
[40] **De Clercq, N., J. Vanden Bussche, S. Croubels, P. Delahaut, and L. Vanhaecke.** 2013. A validated analytical method to study the long-term stability of natural and synthetic glucocorticoids in livestock urine using ultra-high performance liquid chromatography coupled to Orbitrap-high resolution mass spectrometry. Journal of Chromatography A **1301:**111-121.
[41] **Karnes, H. T. and C. March.** 1991. Calibration and Validation of Linearity in Chromatographic Biopharmaceutical Analysis. Journal of Pharmaceutical and Biomedical Analysis **9:**911-918.
[42] **Ewaschuk, J. B., J. M. Naylor, W. A. Barabash, and G. A. Zello.** 2004. High-performance liquid chromatographic assay of lactic, pyruvic and acetic acids and lactic acid stereoisomers in calf feces, rumen fluid and urine. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **805:**347-351.
[43] **Flaherty, J. M., P. D. Connolly, E. R. Decker, S. M. Kennedy, M. E. Ellefson, W. K. Reagen, and B. Szostek.** 2005. Quantitative determination of perfluorooctanoic acid in serum and plasma by liquid chromatography tandem mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **819:**329-338.
[44] **Youssef, Y. A., Y. Beauchamp, and M. Thomas.** 1994. Comparison of a Full Factorial Experiment to Fractional and Taguchi Designs in a Lathe Dry Turning Operation. Computers & Industrial Engineering **27:**59-62.
[45] **Plackett, R. L. and J. P. Burman.** 1946. Biometrika **33:**305-325.
[46] **Box, G. E., and J. S. Hunter.** 1961. 2k-P Fractional Factorial Designs Part I. Technometrics **3:**311-351.
[47] **Eriksson, L., E. Johansson, N. Kettaneh-Wold and S. Wold.** Multi- and Megavariate Data Analysis. Principles and Applications, Umetrics Academy, Umea, 2008.
[48] **Beres, D. L. and D. M. Hawkins.** 2001. Plackett-Burman techniques for sensitivity analysis of many-parametered models. Ecological Modelling **141:**171-183.
[49] **Fedrizzi, B., S. Carlin, P. Franceschi, U. Vrhovsek, R. Wehrens, R. Viola, and F. Mattivi.** 2012. D-optimal design of an untargeted HS-SPME-GC-TOF metabolite profiling method. Analyst **137:**3725-3731.
[50] **deAguiar, P. F., B. Bourguignon, M. S. Khots, D. L. Massart, and R. PhanThanLuu.** 1995. D-optimal designs. Chemometrics and Intelligent Laboratory Systems **30:**199-210.
[51] **Natrella, M. , C. Croarkin, and W. Gutrie.** 2003. Engineering statistics Handbook, NIST/SEMATECH e-

Handbook of Statistical Methods, Gaithersburg, (http://www.itl.nist.gov/div898/handbook/).
[52] **Berg, T. and D. H. Strand.** 2011. C-13 labelled internal standards-A solution to minimize ion suppression effects in liquid chromatography-tandem mass spectrometry analyses of drugs in biological samples? Journal of Chromatography A **1218:**9366-9374.
[53] **Anderson, R. R., D. A. Nixon, and M. A. Akasha.** 1988. Total and free thyroxine and triiodothyronine in blood serum of mammals. Comparative Biochemistry and Physiology A-Physiology **89:**401-404.
[54] **Nixon, D. A., M. A. Akasha, and R. R. Anderson.** 1988. Free and Total Thyroid-Hormones in Serum of Holstein Cows. Journal of Dairy Science **71:**1152-1160.
[55] **Akasha, M. A., R. R. Anderson, M. Ellersieck, and D. A. Nixon.** 1987. Concentration of thyroid hormones and prolactin in dairy-cattle serum and milk at 3 stages of lactation. Journal of Dairy Science **70:**271-276.
[56] **Aceves, C., C. Romero, L. Sahagun, and C. Valverde.** 1987. Thyroid hormone profile in dairy-Cattle Acclimated to cold or hot environmental temperatures. Acta Endocrinologica **114:**201-207.
[57] **Larsson, B., M. Traven, C. Hulten, C. H. Afsegerstad, K. Belak, and S. Alenius.** 1995. Serum Concentrations of Thyroid-Hormones in Calves with a Transient or Persistent Infection with Bovine Viral Diarrhea Virus. Research in Veterinary Science **58:**186-189.
[58] **Tiirats, T.** 1997. Thyroxine, triiodothyronine and reverse-triiodothyronine concentrations in blood plasma in relation to lactational stage, milk yield, energy and dietary protein intake in Estonian dairy cows. Acta Veterinaria Scandinavica **38:**339-348.

CHAPTER VI

General Discussion

and

Future Perspectives

1. GENERAL RESEARCH OUTCOME

1.1. Research positioning

Besides administration for the purpose they were intentionally developed for, i.e. the treatment of 'thyroid rush' or hyperthyroidism in human and veterinary medicine, thyreostatic drugs may be used as illicit weight augmenting drugs in animal production. Several negative aspects have however been attributed to the misuse of these therapeutics including inferior meat quality (water retention), fraud (water sold for the price of meat) and the potential risk of teratogenic and carcinogenic effects from the remaining residues present in animal derived products. This is why the European Union enacted the banishment of thyreostats from meat production in 1981 with a zero-tolerance limit [1].

In order to continuously protect the consumer from potential law violations concerning thyreostat abuse in animal production, a series of strong measures needed to be taken [1]. Amongst those, monitoring methods were developed for thyreostats in thyroid tissue, muscle and urine. At the very beginning, differentiation (compliant/non-compliant) was based on a combination of subjective factors: symptomatology, detection of thyroid hormones, thyroid micro- (histological changes) and macroscopy (weight, goiter). Eventually, the weight shifted towards analytical methodologies for the detection of thyreostats. More recently, the introduction of UHPLC allowed higher sample throughput and increased chromatographic resolution. With this, fast switching QqQ instruments emerged characterized by improved sensitivity and specificity [2-5]. These analytical innovations led to a more reliable detection of thyreostats with increased sensitivity. Clearly, if it was not for these analytical evolutions, the shear existence of natural occurring thyreostats would not have been discovered since these concentrations would simply have remained undetectable. These low-level TU (< 10 µg L^{-1} (RC)) concentrations, which were detected in livestock urine during national control plans, represented a violation of the zero-tolerance limit. However, based on research findings regarding the effect of certain animal feeds (Brassicaceae) on the excretion of thyreostatic compounds in bovine urine [6] and the known kinetics of synthetic thyreostats [7,8] it was hypothesised that the most probable source of endogenous TU was feed-related. Moreover, these low-level TU concentrations had negligible growth promoting potential (growth promoting = 5 g per day p.o.), which further enables the natural occurrence of TU from any potential illegitimate pursue of economical profit.

Because of this, thyreostats were ascribed a dual status, implying either an illicit administration or a feed-related origin.

Within this framework, the scope of this doctoral thesis included to:

- Evaluate the potential of **TU formation upon ingestion** of **Brassicaceae** through *in vitro* digestion simulations of livestock (CHAPTER II)
- **Elucidate the mechanism of intestinal** TU **formation** upon *in vitro* digestion of Brassicaceae feeds (CHAPTER III)
- Isolate and identify the **intestinal bacteria** involved in the formation of TU upon *in vitro* digestion of Brassicaceae feeds (CHAPTER III)
- Optimize the **extraction** of TU from various **animal feeds** and **validate** the analytical method (CHAPTER IV)
- Develop a sensitive and selective UHPLC-MS/MS application for the analysis of **thyroid hormones** in bovine serum to evaluate the effect of 'naturally occurring thyreostats' *in vivo* (CHAPTER V)

1.2. Main research findings

The main accomplishments of this work are summarized in Figure VI.1.

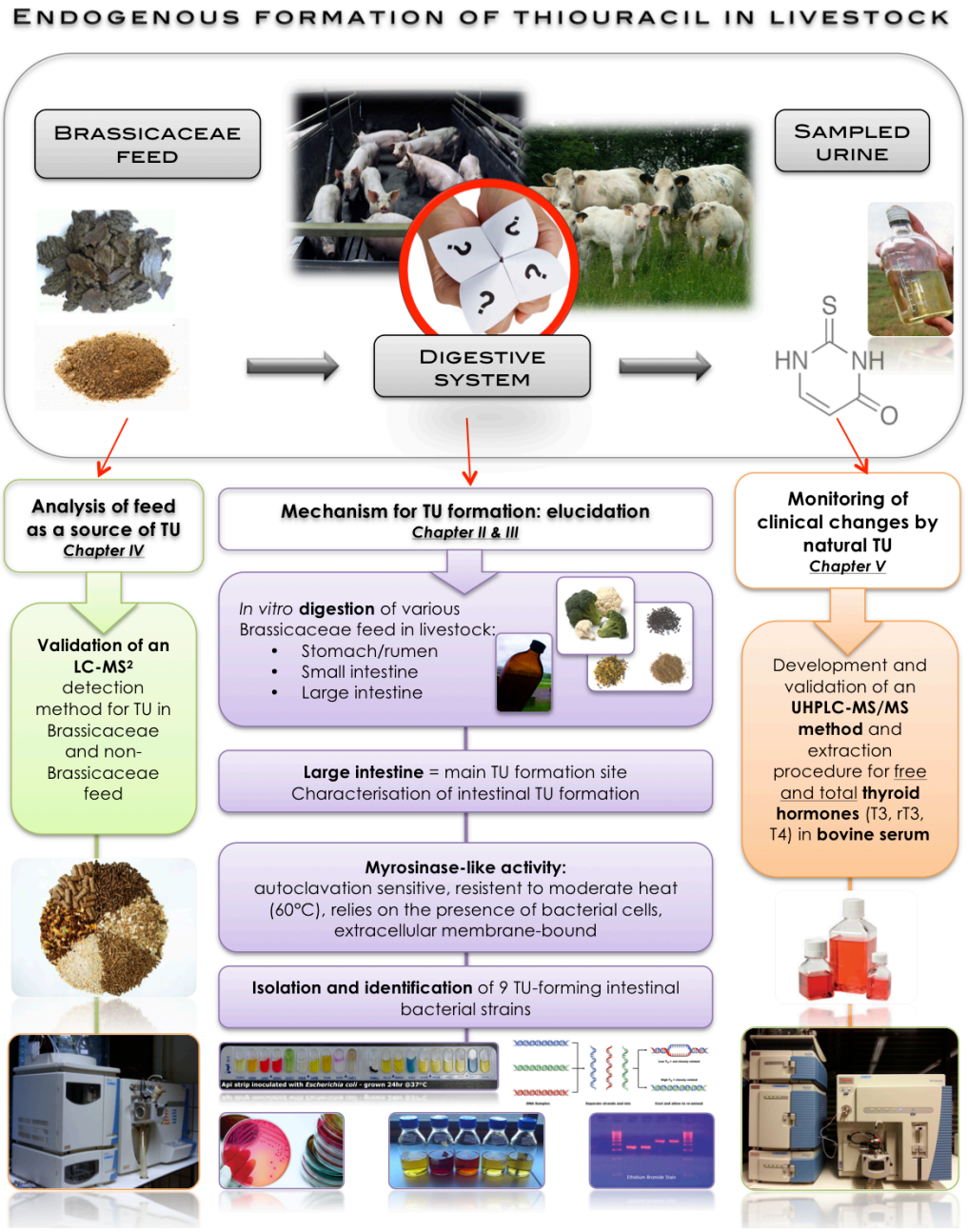

Figure VI.1: Overview of the main realizations achieved in this work.

1.2.1. Elucidation of the mechanism behind thiouracil formation

The terms, natural, semi-endogenous or endogenous TU formation all refer to the same intraorganism formation phenomenon of a non-physiological, at that time alleged synthetic compound, called TU. In previous research low-level TU concentrations have been found in various animal species (e.g. sheep, cattle, horse, dogs and pigs), as well as, humans both with and without diet control [9,10]. Additionally, the ingestion of animal feeds containing (max. 30%) products derived from the Brassicaceae family (cabbage, rapeseed) has been related to the excretion of TU in bovine urine [6]. These findings suggest that the interaction between the ingestion of certain feeds and the metabolisation in the organism causes TU detection. Therefore in **Chapter II** an elaborate evaluation of the TU forming potential of faecal inocula of various animals (bovine and porcine) with addition of specific Brassicaceae feeds (whole rapeseed, grounded rapeseed, cooked rapeseed, coarse colza '00' meal, broccoli or cauliflower) with different pre-treatments (e.g. grounding, cooking) was performed *in vitro*. From this, a clear insight in the formation of TU throughout the digestive tract (stomach/rumen, small intestine and large intestine) was obtained in mono- and polygastric animals. Clearly, the influence of microorganisms in this process was proven, since TU was detected in the most densely populated areas of the GI tract upon Brassicaceae addition, i.e. the colon and to a minor extent the rumen. The concentration of TU detected upon *in vitro* digestion of the different feeds also depended on the GL content. Coarse colza '00' meal for example, with a restricted GL content was consistently the lowest TU producing feed, regardless of the inoculum. Grounding and heating of feeds did not affect TU formation in a colonic *in vitro* digestion. Moreover, a high variability between (bovine and porcine) and within species was found in TU formation. Indeed, a high variability of the intestinal microbial population has been described, even within one species, as many influential factors, like age, feed, physiological status etc. can vary this equilibrium [11-13]. Overall the porcine colonic *in vitro* digestions produced the highest TU concentrations compared to bovines. Therefore, this model was chosen for further experiments to elucidate the mechanism of TU formation.

Furthermore some preliminary experiments are described in **Chapter II**, which intended to characterise the potentially myrosinase-like enzyme activity of colonic suspensions upon *in vitro* digestions with rapeseed. These experiments confirmed the involvement of bacteria in the formation of TU during *in vitro* Brassicaceae digestions. Filter sterilization and autoclavation of the not pre-cultured faecal inoculum showed

a significant decrease of TU formation during subsequent *in vitro* Brassicaceae digestions. At this stage however, the hypothesis of a non-myrosinase-like bacterial precursor was briefly envisaged based on the fact that in pre-cultured inocula, TU formation after filter sterilisation was increased, which was not the case when non-pre-cultured inocula were used. This finding pointed towards a potential non-cellular activity, which could not be related to myrosinase-like enzymatic activity, since bacterial myrosinase had been reported to possess a cell-dependent activity [14,15]. Furthermore, the incubation of Brassicaceae colonic *in vitro* digestion suspensions at the end of the digestion with sinigrin, a GL, was not capable in producing glucose, although addition of synthetic myrosinase (purified from mustard seed, *Sinapsis alba* L.) did induce sinigrin hydrolysis with subsequent glucose formation. This purified plant-derived myrosinase from *Sinapsis alba* L. could degrade sinigrin since this is the second most abundant GL within white mustard seeds (sinalbin > sinigrin) [16]. In rapeseed other glucosinolates (progoitrin, gluconapin and 4-hydroxy-glucobrassicin) are dominant [16,17] than sinigrin [17,18]. As such, it was however too precarious to conclude that the suspected TU forming myrosinase-like bacterial activity is not present because of different glucose production compared to the activity of the plant-derived myrosinase of mustard seed. The bacterial myrosinase activity, which is also able to form TU, is potentially more adapted to the GLs abundant in rapeseed. This has also been reported for degradation of GLs by intestinal bacteria, showing that pre-culturing bacteria in a sinigrin enriched medium induced their subsequent sinigrin degrading activity rate compared to control cells grown on glucose [19]. Furthermore, the Brassicaceae family contains a variety of myrosinase enzymes. However, not much is known about their properties or the role of their iso-enzymes [20,21]. In this light, it is likely that also different types of bacterial myrosinase-like enzymes can be formed and that proving the myrosinase-like hypothesis with indirect detection methods will also depend on the physico-chemical characteristics of the bacterial equivalent.

A more in depth evaluation of the mechanistical background of TU formation in **Chapter III,** included amongst others proof that the presence of bacteria was prerequisited by double filter sterilisation, which similarly confirmed the previous results, showing a decrease in TU. Trough pasteurisation it was shown that TU could be formed upon treatment of the bacterial inoculum with moderate heat (60 °C) for a specific (max. 30 min) period of time, after which the effect seemed lost. Although heat-treated microorganisms generally do not grow on agar, they can succeed in repairing damage and regain their potential for growth under optimal broth

conditions [22]. Such events might explain the undisturbed TU production over the first 30 minutes of pasteurisation, while after 60 min bacterial survival is unlikely due to irreversible damage. Inactivation of certain plant-derived myrosinases also shows resistance to a range of temperatures (30-65 °C (for rapeseed)) depending on the crop after which inactivation sets in [23]. Moreover, it has been described that certain proteins can regain activity after moderate heat inactivation [24]. Besides, *E. coli* has been reported to survive up to 30 min at 60 °C, dependent on strain, medium and optimal conditions, which is comparable to the present results [22]. At 55 °C, inactivation is only achieved after 60-120 min in broth [22]. Therefore, in the assumption that the responsible (bacterial) mediator is a membrane-bound protein (myrosinase-like enzyme), heat treatment would likely cause some degree of denaturation over time, but semi-reversible denaturation has been reported [24]. Moreover, β-thioglucoside glucohydrolases remain active in a broad range of temperatures (up to 60 °C) and pH (5-10) [25] offering an explanation for sustained TU production upon pasteurization and decreased TU production upon double filter sterilisation, thereby supporting the hypothesis of an extracellular membrane-bound myrosinase-like enzyme as mediating factor for TU formation.

As a second part of elucidating the mechanism of TU formation, in **Chapter III,** TU forming bacteria were successfully isolated and identified from rapeseed colonic digestion simulations of porcines. Besides, a higher rate of intestinal TU formation was noted under anaerobic conditions. To the best of our knowledge, no previous reports have been published describing the identification of TU forming bacteria in porcines. The nine common intestinal bacteria that were identified belonged to 4 different species (*E. coli, E. faecium, L. reuteri* and *Salmonella enterica ssp. arizonae*) and provide a first insight into the potential actors of the endogenous TU formation process. A β-glucosidase GLs degrading myrosinase-like enzymatic activity has been described for all the identified bacterial species [19,26-31]. Strains of the genera of *Escherichia, Lactobacillus* and *Bifidobacterium* have been reported to produce surface-associated extracellular proteins/peptides [32]. Interestingly, these bacteria are common intestinal bacteria in both porcines and bovines [33-40].

With respect to rapeseed as a source of TU, autoclavation thereof caused an insignificant decrease in TU formation compared to a positive non-autoclaved rapeseed control when incubated with pre-cultured faecal inoculum, in **Chapter II**. The postulated explanation for this was that faecal debris present in the inoculum can act as precursor for TU when no rapeseed is added. In **Chapter III**, this was confirmed

since the addition of rapeseed to faecal inoculum did not increase TU formation significantly as compared to a control. Indeed, in literature it has been described that 60% of the GL content of feed reaches the colon unaltered in pigs and although these are mostly degraded prior to defecation, many breakdown products are present in faeces [41]. When only autoclaved rapeseed (**Chapter III**) was incubated, TU formation was negligible. When however non-autoclaved rapeseed was incubated again without faecal inoculum a slight increase of TU was observed in comparison with autoclaved rapeseed. *In vitro* degradation of the aliphatic glucosinolates sinigrin, progoitrin and gluconapin as well as the aromatic glucosinolate sinalbin by phytophatogenic fungi of Brassica has been described [42]. But, because these TU increases never exceeded the background level of TU in 24 h pre-cultured faecal inoculum, it is highly unlikely that contamination would significantly contribute to endogenous TU formation, as previously stated (**Chapter II**). Next, to further confirm Brassicaceae were in fact needed for TU formation, blank, autoclaved rapeseed and rapeseed incubations were performed with two TU producing strains, i.e. *L. reuteri* and *E. faecium*, thereby omitting the influence of degraded GL faecal constituents. Since addition of rapeseed as a whole did significantly increase TU formation upon incubation with pure strains, its importance as a source of TU precursor in line with previous *in vivo* [6] research was confirmed. Furthermore, the enumeration of pure strains *E. coli*, *L. reuteri* and *E. faecium* showed no growth inhibiting effects due to rapeseed [43] addition in the *in vitro* simulations.

From **Chapter II** the inter- and intra-species variation of *in vitro* intestinal TU formation became clear. When, however, evaluating the relation between the age of the animals and TU detection in livestock urine, literature has indicated that young bovines have been reported to have higher concentrations of TU in their urine in comparison to adult cattle and porcines [44-46]. In the present study the TU forming activity in porcine *in vitro* colonic digestions was observed to increase with age (**Chapter III**). Taking into consideration several threshold studies [44-46] it should be noted that these only inquired the presence of TU in urine of young porcines. Therefore, the relatively low TU concentrations in porcines might be more related to the selected subgroup (1-8 months) and only to a lesser extent to the effect of rapeseed in their diet [44,45]. Moreover, taking into account the newly identified TU producing bacterial strains in porcines, this finding is also supported by scientific literature, since these specific bacterial strains are more present in young calves (high TU producing) and older porcines, whereas less in young porcines [39,34,47,48].

In conclusion, the anaerobic process of TU formation was tentatively characterised as an interaction between rapeseed (Brassicaceae) and bacteria, with the latter producing a membrane-bound extracellular enzyme sustaining moderate heat, but not autoclavation expressing a glucosinolate (myrosinase-like) degrading activity.

However, to be able to extrapolate and confirm these *in vitro* findings there is need for *in vivo* testing. The concentrations detected *in vitro* are based on the interaction between high glucosinolate content traditional rapeseed and inocula or bacterial strains, without intestinal cell wall interactions (absorption, secretion, etc.). These values can therefore not unilaterally be transposed to the live situation in the animal since mostly low glucosinolate feeds are commonly used nowadays. However, these results do show intestinal transformation is possible based on the interaction between certain bacteria and Brassicaceae feedstuffs. Also, the intestinal formation process has been shown to be susceptible to certain factors (oxygenation, heat, becteria etc.). An *in vivo* study would enable further evaluation of the process, showing if feeding low glucosinolate Brassicaceae (max. 30%) based feedstuffs upon digestion is the sole inducer of TU occurrence in livestock urine. More importantly, it could potentially demonstrate if TU formed intestinally upon Brassicaceae digestion and TU administration, indepently, have a different influence on TU concentrations but also the presence of potential biomarkers in livestock urine. Besides, absorption and excretion kinetics of TU, as well as, thyroid hormone patterns could be evaluated in parallel with the evaluation of other influential factors (e.g.: age, gender, species). At the moment such an *in vivo* study is on going at our laboratory, called the THYREOMERK FOD-project (cfr. 1.3 *Future perspectives*).

1.2.2. Feed analysis developments

The main goal of **Chapter IV** involved the optimization of a robust detection method for TU in various animal feedstuffs from Brassicaceae and non-Brassicaceae origin. Literature reports are very scarce regarding specific methodologies for thyreostat analysis from animal feedstuffs [6,49-51]. The developments for thyreostat detection by Pinel *et al.* (2005) [2] on a triple quadrupole mass spectrometer in rapeseed and cabbage did not enable TU detection. It was only when the method was adapted by Vanden Bussche *et al.* (2011) [52], who added a hydrolysis step with plant-derived myrosinase to the extraction procedure that the presence of TU could be discovered in various Brassicaceae vegetables, seeds and meals. In this research, the extraction protocol was optimized for both Brassicaceae and non-Brassicaceae feedstuffs, since both are being administered to animals but little to no information is available

regarding their TU status. Surprisingly, after statistical Plackett-Burman optimization myrosinase was ruled out as a major influential factor in the detection of TU in Brassicaceae and other feedstuffs. This confirmed a preliminary result, which showed adding various amounts (0-3 mL) of myrosinase to the feed extraction procedure from Vanden Bussche *et al.* (2011) [52] decreased TU detection from coarse colza '00' meal. The influence of sonication, over night incubation of the feed in a pH 7 buffer and the optimization of the extraction steps are probably much more important and make the addition of plant-myrosinase superficial. Whether the detected thiouracil is originating from TU present in the feed and/or TU formation based on a precursor cannot be determined. It is, however, not unthinkable that during the incubation of feed in buffer solution residual myrosinase activity (plant or microbial) might cause slight TU formation.

The LOD of 0.2 µg TU kg^{-1} achieved by this method is well below the 10 µg kg^{-1} RC, and the previous 150 µg kg^{-1} achieved by the method described by Pinel *et al.* (2005) [2]. To the best of our knowledge this is the first method that was successfully validated for TU in feedstuffs and proved applicable for different types of fodders, obtained through the national control plan of Ireland upon positive urine testing for TU.

Method developments for thyreostats in animal foodstuffs, have gained increasing attention lately, as the latest 2014 Reflection paper (Natural growth promoting substances in biological samples) by the European Reference Laboratories included feed analysis as part of the control plan [53]. This paper stated that undoubtable low-levels of TU that occasionally are detected in samples of urine are the result of TU precursors present in animal feed. Moreover, since several studies independently [53,44-46] indicate that urinary TU levels can exceed the current RC of 10 µg L^{-1}, it was suggested to increase the RC up to 30 µg L^{-1}. Indeed, research showed [53] that the administration of TU with a double daily dose of 2.5 g p.o. in cattle displayed mg L^{-1} instead of µg L^{-1} concentrations of urinary TU (Figure I.2), which is the dosage (5 g per day) that has to be administrated to observe the expected weight gain, illustrating the fact that higher (ppm-range) urinary levels should be expected upon illicit administration. On that account pleading for a higher TU limit (30 µg TU L^{-1}) seems defendable [44].

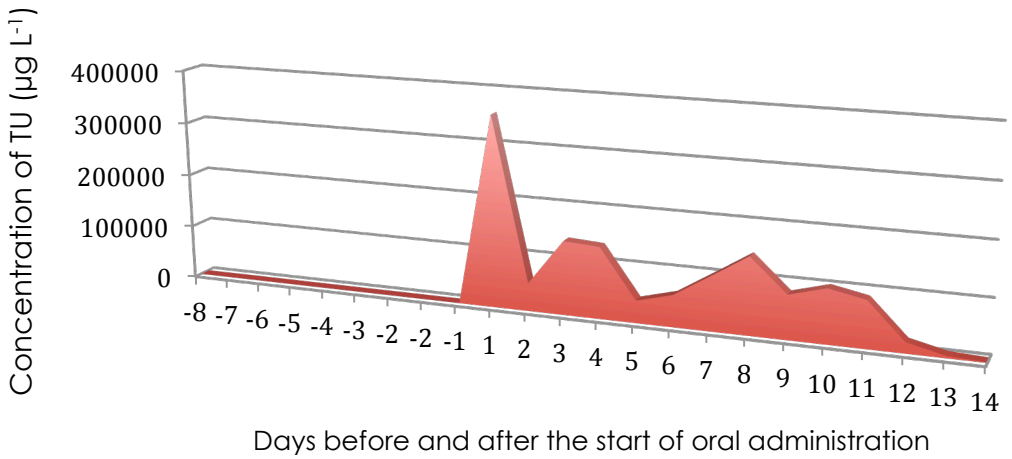

Fig VI.2: Excretion of thiouracil in urine over a 14-day period in a young heifer, which was administered 5 g thiouracil per day (2*2.5 g p.o.) [42].

In case this new urinary RC is exceeded, a new strategy is proposed in the Reflection paper, including the analysis of the administered feed with and without myrosinase hydrolysis as reported by Vanden Bussche *et al.* (2011) [52]. A flowchart (Figure I.3) shows the mode of action to either confirm or refute that feed is the source of TU detection in urine. When no direct link can be established between TU in urine and feed samples, follow-up investigations on the farm are required. Lastly, the stabilisation (pH=1, EDTA) [54] of urine samples and minimisation of freeze-thaw cycles is also encouraged since reports have shown detection can thereby be impaired, resulting in underestimation of thyreostat concentrations [4,53].

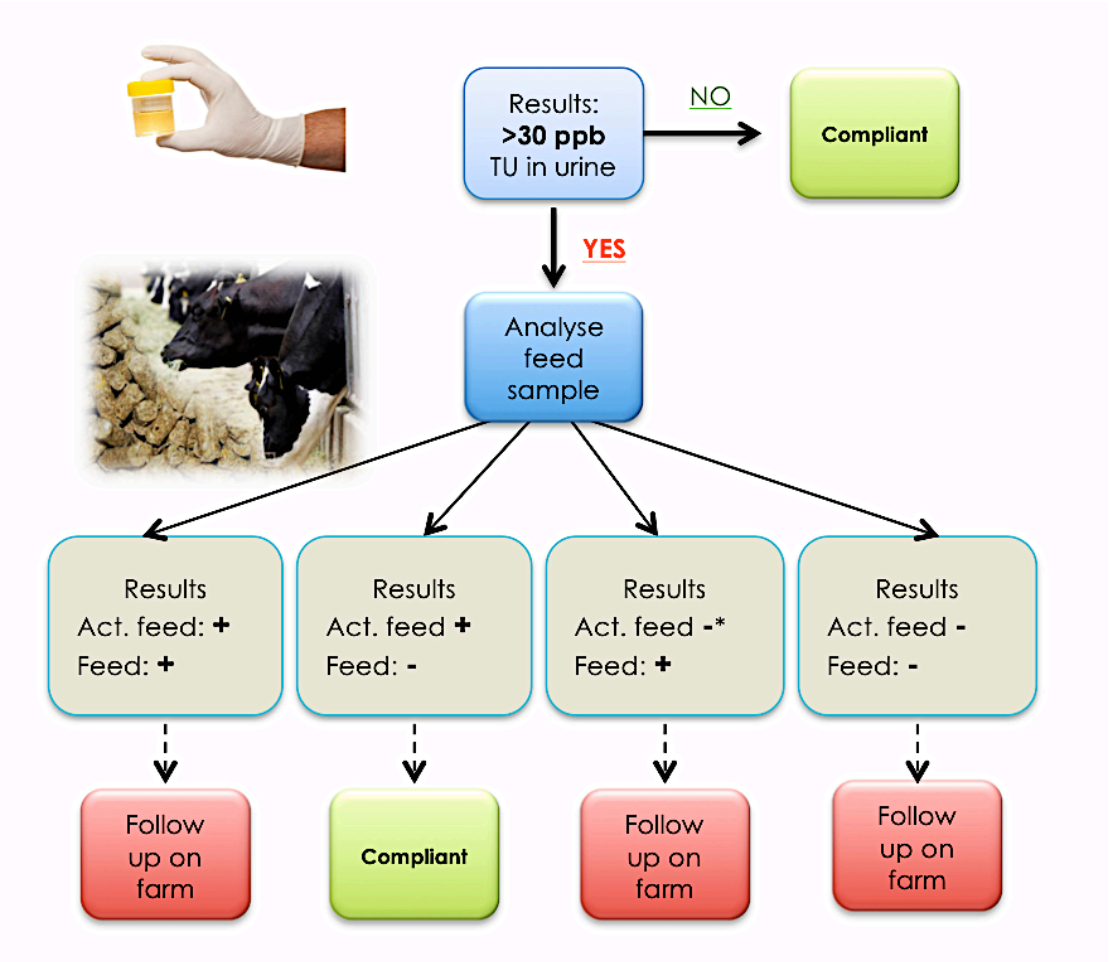

Figure VI.3: Decision flow chart for thiouracil findings in bovine urine. Feed was extracted with ('Act. Feed') and without ('Feed') myrosinase addition [53]. (*: After activation no significant increase in thiouracil concentration was seen)

1.2.3. Thyroid hormone analysis developments

Thyroid hormones are essential to sustain a normal functioning metabolism, even though the thyroid gland is one of the smaller organs of the organism. These hormones influence a variety of pathways and organs e.g. the metabolic rate (carbohydrate and lipid metabolism), growth, the cardiovascular, the reproductive and the central nervous system [55]. Therefore, the assessment of thyroid hormone is an essential parameter in the clinical evaluation of thyroidal disorders in animals and humans.

The analysis of thyroid hormones, however, presents many challenges. Firstly, the fact that thyroid hormones can be either bound to transporter proteins, or remain free in the bloodstream, while maintaining an equilibrium, which is an important parameter in diagnostics. Therefore, this physiological equilibrium (free:bound) should be

reflected by analysis without suffering from potential deviations mainly inflicted by inappropriate sample preparation conditions. Secondly, the analysis itself might be a challenge since concentrations can go down to low ppt-levels (e.g. for free thyroid hormones in animals). For that reason, a powerful and sensitive detection tool is necessary. Third is the sample preparation and subsequent extraction that needs to be adapted to the fraction (free or bound), which will be analysed. The most common factors, that have been described to exert an influence on the final results, are e.g. the use of serum separator tubes, time interval prior to centrifugation, centrifugation temperature [56], effect on whole blood, serum and plasma of storage temperature and time [57]. Besides, endocrine diagnostic testing can be affected by many clinical factors (medication, illness, stress concurrent illnesses and individual characteristics (age, breed, gender and reproductive status). Therefore, by minimizing the effect of sample handling and treatment at least the non-physiological variability can be omitted, increasing the correlation between the results and the physiological state of the animal, which in turn improves the reliability of the final diagnostic.

The thyroid hormone analysis method (**Chapter V**) was developed in agreement with previous scientific reports with respect to sample pre-treatment. For example, serum was obtained from fully clothed whole blood which was directly centrifuged and serum samples were stored at -20 °C, freeze-thaw cycles were minimized, centrifugation temperatures approximated body temperature and extracted samples were analysed the same day [56-58]. Moreover, since most thyroid hormone detection methods used in veterinary medicine rely on ELISA, RIA or alternatively CLA [59,60], the present method, using mass spectrometry as a detection technique, is unique for veterinary purposes. Although MS had been already broadly introduced and even accepted as a golden standard in human thyroid hormone analysis [56,58], no reports on veterinary applications were available. Furthermore, the UHPLC-MS/MS method was sensitive enough to analyse both free and total thyroid hormone samples after different extraction procedures were applied. Total thyroid hormone included deproteinisation and liquid-liquid extraction whereas free thyroid hormone needed separation through ultrafiltration and solid phase extraction. For the separation of the free hormone fraction ultrafiltration was elected above equilibrium dialysis, because the latter is commonly used in animals but proves to be more labour intensive and costly [56].

Ultimately, the goal of the development of this thyroid hormone UHPLC-MS/MS method is to evaluate the effect of endogenous TU formation in livestock, based on the ingestion of different diets. Within the THYREOMERK FOD-project (RF12 6260), this

method will be used to evaluate the thyroid status of animals under various Brassicaceae and non-Brassicaceae diets (cfr. *1.3 Future perspectives)*.

Finally, with respect to the thyroid hormone detection method, we believe it could easily lend itself to different other species, beside bovines, broadening its field of application in veterinary medicine, considering minor optimizations would be performed.

1.3. Future perspectives

Despite of the findings reported in this study with respect to the endogenous status of TU, some questions still remain. Potential research paths may amongst others include discovering a biomarker that allows differentiating endogenous formation from exogenous administration of thyreostats, further investigation of potential other feed sources that sustain TU formation, evaluation of the risk of low-level natural TU through consumption *in vivo*. Eventually, all these will provide a strong base for an adapted legislative framework. Notwithstanding, derogations and adaptation of the EU legislation may already be made based on the current results and knowledge.

1.3.1. Discriminating endogenous from exogenous thiouracil

At the time, a **direct discrimination** between endogenous and exogenous TU has not yet been achieved. The investigation of a potential biomarker that would render differentiation possible is still on-going e.g. the THYREOMERK FOD-project. This project is a cooperation between our laboratory (Laboratory for Chemical Analysis) and the CER (Centre d'Economie Rurale, Marloie, Belgium), aiming amongst others to set a threshold for TU in urine based on the thorough statistical evaluation of control plan data from national reference laboratories all over Europe (e.g.: Belgium, Ireland, Norway, Poland, Netherlands). This will allow a more accurate differentiation between compliant and non-compliant urine samples. Moreover, it will evaluate the effect of administering thiouracil, feeding Brassicaceae and non-Brassicaceae fodders in a bovine *in vivo* study on the basis of TU detection (urine, feed) and thyroid hormone detection (serum). Eventually, the *in vivo* collected urine will be pivotal to finding a specific biomarker for the differentiation of endogenous TU formation based on an untargeted high-resolution mass spectrometry (HRMS) approach. Several types of HRMS techniques exist with variable resolving power, for example ToF with 10 000-40 000 full width half maximum (FWHM), the Fourier transform ion cyclotron resonance

(FT-ICR) and Fourier transform Orbitrap MS with usually 100 000-140 000 at 200 *m/z* and for the latest generation more than 240 000 at 400 *m/z* can be achieved [61,62]. The use of HRMS is highly encouraged for biomarker investigations, as these techniques posses besides this high resolving power, also high mass accuracy, sensitivity, dynamic range and make post acquisition data mining possible [63,64]. This latter aspect is quite interesting as full scan spectra of matrices from control, TU treated, and rapeseed fed animals could be evaluated untargetedly. This type of approach requires a highly generic sample preparation in order to preserve as many unknown components as possible [65]. For now however, only one targeted HRMS application has been described in bovine urine by Léon *et al.* (2012) [66] for a multi-residue analysis of 87 banned or non-regulated veterinary drugs (a.o. steroid hormones, β-agonists, resorcylic acid lactones (RAL), stilbens, tranquillizers, nitroimidazoles, corticosteroids, NSAIDs, amphenicoles, thyreostats, etc.), including thyreostats. Léon *et al.* (2012) [66] applied a generic approach identical for all components called QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), which was based on buffered extraction with acetonitrile and dispersive-SPE, achieving detection for all components with a mass deviation less than 5 ppm at 50,000 FWHM. Except for 6-phenyl-2-thiouracil, the method reached a CC_β of 10 µg L^{-1} for all considered thyreostats (including TU), which is equal to the RC. According to the CD/2002/657 [67] the CC_β should be below the RC, which is also indicated for detection of low-level concentration of TU in urine originating from animal feed. Further optimization in this area would therefore be advisable.

In the case of natural thiouracil differentiation, a new untargeted method remains to be developed. If successful this type of approach will need the assistance of specific mathematical algorithm-based statistical data mining software due to the large amount of acquired spectral data in HRMS. These types of software can perform trend (multivariate) analysis to identify compounds (*m/z*) that vary with dosage, time, or other trend classifications (e.g. TU administration or Brassicaceae feed administration). Statistically rigorous data mining tools have the power to compare hundreds of LC/MS data files at once, by prefiltering complex data, greatly reducing the number of compounds that need to be evaluated and significantly decreasing time spent on identification. The latter can be performed using public domain user-selected databases. Plot visualization of the data can help discriminate the significantly influential *m/z* values from the less influential and disclose certain patterns. Besides, identification of the potential biomarker metabolite of interest, quantitative method validation should also be conducted evaluating the performance of the method, similarly to the well-known evaluation parameters

described in the CD/2002/657 [67] (e.g. sensitivity, precision, recovery). Untargeted approaches typically use relative quantification (i.e. reporting of metabolite(s) instrument response relative to an internal standard or another metabolite(s) level such as the sum of all metabolite abundance). Whereas, absolute quantification (determination of the absolute concentration of a metabolite) through correlation of its instrument response to that of a known concentration series of the same metabolite is commonly used in targeted metabolite(s) analysis [68].
Furthermore, these HRMS techniques, like Orbitrap and ToF, can be hyphenated with an ion trap (e.g. LTQ-Orbitrap) or a triple quadrupole (Q-Exactive, QqToF) allowing structural elucidation and identification of the potential biomarker/metabolite through fragmentation. Regarding quantification, 4 IPs are required for group A substances according to the CD/2002/657 [67] and each HRMS ion earns a minimum of two IPs, therefore two molecular ions (HRMS) (often deliberately combined with C^{13}/C^{12} ratio's) or one quantitative molecular ion and one confirmatory product ion (hybrid HRMS) suffice to confirm any substance [62].

Once biomarkers for differentiation of endogenous TU would be available, evaluation of these biomarkers in other matrices (besides urine), which would have been confirmed with TU presence (e.g. thyroid), should be performed as well. Although it is not certain that the urine biomarker would automatically be present in other matrices. Potential matrices should be foremost easy to sample and might therefore include faeces, but also saliva or even breath. In the latter case natural TU would need to cause specific volatile organic compounds (VOCs) to be exhaled in breath, which would be significantly different from exogenous administration. In humans, research on breath has been performed for pre-clinical disease markers [69], but lately also in bovines disease differentiation based on VOCs has been proven [70]. It remains however to be seen whether a discriminating biomarker is present in other matrices then urine for natural TU. The practical on-site biomarker evaluation of a subset of animals would then be possible if portable sensitive and selective devices were available, which would make evaluation of the animals quicker, less time and labour intensive. This type of analysis, however, has still a long 'optimization road' to go before a practical application can be foreseen. Notwithstanding, the combination of a preliminary biomarker screening method and confirmatory mass spectrometrical analysis could be foreseen in this way. Such combination has already been put forward for hormone abuse in cattle [71].

Regarding the applied methodology for the endogenous TU discriminating biomarker quest, other methods besides HRMS, like isotope ratio MS, may also be considered in the differentiation of endogenous versus exogenous TU. This approach enables precise measurements of stable isotope ratios ($^{12}C/^{13}C$) within a sample resulting from fractionation patterns inherent to the endogenous or exogenous origin of the compound. This procedure has been widely used in the differentiation of steroids [63,72-74] and has been lately put forward for evaluation of its applicability in the differentiation of corticosteroids [53].

1.3.2. Legislation: is an adaptation required?

Based on the current **legislation** surrounding thyreostat abuse, it may be stated that certain actions should be taken to resolve the duality concerning thyreostats. The current zero-tolerance [1] for thyreostats in animal matrices is still held on to by way of precaution, but at this time the dual status of TU has been proven [6,9,10,52,75], which should also be acknowledged by the EU by providing a more adapted legislative framework. Future European legislation should, therefore, include derogation clauses for TU detection coming from an endogenous source (feed-related). Since the RC currently at hand [76] has no power of law, it would be opportune that the EU would adopt a TU reference concentration (or threshold) below which TU is accepted to be of endogenous origin in order to clarify the present situation. The proposition to develop a reference point for action (RPA) (Council Regulation (EC) No 470/2009) [77] was a step in the good direction, but the actual elaboration of an RPA level for thyreostats and its implementation is still awaited.

Moreover, with the increasing amount of threshold research regarding thyreostats [44-46], setting a RPA or threshold value should be feasible on a country-based level. Thresholds values for TU in urine samples were proposed at 95% and 99% for France amounting at 5.7 $\mu g.L^{-1}$ and 9.1 $\mu g.L^{-1}$ in male adult bovines (6-24 months), 3.1 $\mu g.L^{-1}$ and 8.1 $\mu g.L^{-1}$ in female adult bovines (6-24 months), 7.3 $\mu g.L^{-1}$ and 17.7 $\mu g.L^{-1}$ in calves (< 6 months), 3.9 $\mu g.L^{-1}$ and 8.8 $\mu g.L^{-1}$ in female adult bovines (> 24 months), 2.9 $\mu g.L^{-1}$ and 4.1 $\mu g.L^{-1}$ in porcines (1-8 months) [44]. Poland had 95th and 99th percentiles for TU below 4.50 and 14.85 $\mu g\ L^{-1}$ for bovine urine samples and below 2.35 and 6.80 $\mu g\ L^{-1}$ for porcine samples. Even though, the results of both countries were concurrent a country-based assessment seems advisable, in order to potentially discriminate inter-country variations [45].

1.3.3. Application of analytical developments

Finally, the **analytical methods developed** in this study for thyroid hormones in bovine serum and TU in animal feed could be used for scientific purposes.

The evaluation of clinical changes concerning the thyroid status and the effect of endogenous/exogenous TU could be evaluated. The elaboration of the thyroid hormone detection method to other species should be pursued in order to create an alternative for current detection in veterinary medicine.

The detection method for TU in Brassicaceae and non-Brassicaceae feed could also be used as a screening tool for animal feeds before administration (upon manufacturing) or as a control parameter when a positive urine sample is encountered in order to trace the origin back to the ingested feed. Additionally, a broad evaluation of a large variety of animal feeds with known compositions and known-manufacturing processes could clarify and potentially lead to classification of animal feeds according to their potential to form TU *in vivo*, which should be confirmed in live animals. In general practice, it is not feasible to link back TU positive urines to the originally ingested feed. Consequently, parallel ran samplings of feed and urine could make a closer follow up possible [53].

2. REFERENCES

[1] **European Community.** 1981. Council Directive 81/602/EC. Official Journal of the European Community **L 222**:32-33.

[2] **Pinel, G., E. Bichon, K. Pouponneau, D. Maume, F. Andre, and B. Le Bizec.** 2005. Multi-residue method for the determination of thyreostats in urine samples using liquid chromatography coupled to tandem mass spectrometry after derivatisation with 3-iodobenzylbromide. Journal of Chromatography A **1085:**247-252.

[3] **Vanden Bussche, J., H. Noppe, K. Verheyden, K. Wille, G. Pinel, B. Le Bizec, and H. F. De Brabander.** 2009. Analysis of thyreostats: A history of 35 years. Analytica Chimica Acta **637:**2-12.

[4] **Vanden Bussche, J., L. Vanhaecke, Y. Deceuninck, K. Verheyden, K. Wille, K. Bekaert, B. Le Bizec, and H. F. De Brabander.** 2010. Development and validation of an ultra-high performance liquid chromatography tandem mass spectrometry method for quantifying thyreostats in urine without derivatisation. Journal of Chromatography A **1217:**4285-4293.

[5] **Lega, F., L. Contiero, G. Biancotto, and R. Angeletti.** 2013. Determination of thyreostats in muscle and thyroid tissues by QuEChERS extraction and ultra-performance liquid chromatography tandem mass spectrometry. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment **30:**949-957.

[6] **Pinel, G., S. Mathieu, N. Cesbron, D. Maume, H. F. De Brabander, F. Andre, and B. Le Bizec.** 2006. Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyrostats in meat production. Food Additives and Contaminants **23:**974-980.

[7] **De Brabander, H.F.** 1984. Bepalingsmethoden voor thyreostatica in biologisch material. Thesis, Ghent University, Belgium.

[8] **Heeremans, A., Ermens, L., De Wash K., Van Peteghem, C., De Brabander, H.F.** 1998. Elimination profile of methylthiouracil in cows after oral administration. The Analyst **123:** 2629-2632.

[9] **Pinel, G., D. Maume, Y. Deceuninck, F. Andre, and B. Le Bizec.** 2006. Unambiguous identification of thiouracil residue in urine collected in non-treated bovine by tandem and high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry **20:**3183-3187

[10] **Vanden Bussche, J., L. Vanhaecke, Y. Deceuninck, K. Wille, K. Bekaert, B. Le Bizec, and H. F. De Brabander.** 2011. Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry detection of naturally occuring thiouracil in urine of untreated livestock, domesticated animals and humans. Food Additives and Contaminants **28:**166-172.

[11] **Jensen, M. T., R. P. Cox, and B. B. Jensen.** 1995. Microbial-Production of Skatole in the Hind Gut of Pigs Given Different Diets and Its Relation to Skatole Deposition in Backfat. Animal Science **61:**293-304.

[12] **Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon.** 2008. Evolution of mammals and their gut microbes. Science **320:**1647-1651.

[13] **Saric, J., Y. Wang, J. Li, M. Coen, J. Utzinger, J. R. Marchesi, J. Keiser, K. Veselkov, J. C. Lindon, J. K. Nicholson, and E. Holmes.** 2008. Species variation in the fecal metabolome gives insight into differential gastrointestinal function. Journal of Proteome Research **7:**352-360.

[14] **Palop, M. L., J. P. Smiths, and B. Tenbrink.** 1995. Degradation of Sinigrin by Lactobacillus-Agilis Strain R16. International Journal of Food Microbiology **26:**219-229.

[15] **Cheng, D. L., K. Hashimoto, and Y. Uda.** 2004. In vitro digestion of sinigrin and glucotropaeolin by single strains of Bifidobacterium and identification of the digestive products. Food and Chemical Toxicology **42:**351-357.

[16] **Ciska, E., J. Honke, and H. Kozlowska.** 2008. Effect of light conditions on the contents of glucosinolates in germinating seeds of white mustard, red radish, white radish, and rapeseed. Journal of Agricultural and Food Chemistry **56:**9087-9093.

[17] **Li, Y. C., G. Kiddle, R. Bennett, K. Doughty, and R. Wallsgrove.** 1999. Variation in the glucosinolate content of vegetative tissues of Chinese lines of *Brassica napus* L. Annals of Applied Biology **134:**131-136.

[18] **Velasco, P., P. Soengas, M. Vilar, M. E. Cartea, and M. del Rio.** 2008. Comparison of glucosinolate profiles in leaf and seed tissues of different *Brassica napus* crops. Journal of the American Society for Horticultural Science **133:**551-558.

[19] **Palop, M. L., J. P. Smiths, and B. Tenbrink.** 1995. Degradation of sinigrin by *Lactobacillus Agilis* Strain R16. International Journal of Food Microbiology **26:**219-229.

[20] **Rask, L., E. Andreasson, B. Ekbom, S. Eriksson, B. Pontoppidan, and J. Meijer.** 2000. Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Molecular Biology **42:**93-113.

[21] **Andreasson, E., L. B. Jorgensen, A. S. Hoglund, L. Rask, and J. Meijer.** 2001. Different myrosinase and idioblast distribution in *Arabidopsis* and *Brassica napus*. Plant Physiology **127:**1750-1763.

[22] **Usajewicz, I., and B. Nalepa.** 2006. Survival of *Escherichia coli* O157:H7 in milk exposed to high temperatures and high pressure. Food Technology and Biotechnology **44:**33-39.

[23] **Van Eylen, D., Indrawati, M. Hendrickx, and A. Van Loey.** 2006. Temperature and pressure stability of mustard seed (*Sinapis alba* L.) myrosinase. Food Chemistry **97:**263-271.

[24] **Dewit, J. N., and G. Klarenbeek.** 1984. Effects of various heat-treatments on structure and solubility of whey Proteins. Journal of Dairy Science **67:**2701-2710.

[25] **Zhou CH, Tokuhisa JG, Bevan DR, Esen A.** 2012. Properties of beta-thioglucoside hydrolases (TGG1 and TGG2) from leaves of *Arabidopsis thaliana*. Plant Science **191:**82-92.
[26] **Oginsky, E. L., A. E. Stein, and M. A. Greer.** 1965. Myrosinase activity in bacteria as demonstrated by conversion of progoitrin to goitrin. Proceedings of the Society for Experimental Biology and Medicine **119:**360-4.
[27] **Nugon-Baudon, L., S. Rabot, J. M. Wal, and O. Szylit.** 1990. Interactions of the intestinal microflora with glucosinolates in rapeseed meal toxicity - 1st Evidence of an intestinal *Lactobacillus* possessing a myrosinase-like activity *in vivo*. Journal of the Science of Food and Agriculture **52:**547-559.
[28] **Rabot, S., L. Nugonbaudon, P. Raibaud, and O. Szylit.** 1993. Rapeseed meal toxicity in gnotobiotic-rats - Influence of a whole human fecal flora or single human strains of *Escherichia coli* and *Bacteroides vulgatus*. British Journal of Nutrition **70:**323-331.
[29] **Li, F., M. A. J. Hullar, Y. Schwarz, and J. W. Lampe.** 2009. Human gut bacterial communities are altered by addition of cruciferous vegetables to a controlled fruit- and vegetable-free diet. Journal of Nutrition **139**:1685-1691.
[30] **Mroczynska, M., and Z. Libudzisz.** 2010. Bèta-glucuronidase and bèta-glucosidase activity of *Lactobacillus* and *Enterococcus* isolated from human feces. Polish Journal of Microbiology **59**:265-269.
[31] **Mullaney, J. A., W. J. Kelly, T. K. McGhie, J. Ansell, and J. A. Heyes.** 2013. Lactic acid bacteria convert glucosinolates to nitriles efficiently yet differently from *Enterobacteriaceae*. Journal of Agricultural and Food Chemistry **61:**3039-3046.
[32] **Sánchez, B., M. C. Urdaci, and A. Margolles.** 2010. Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa-bacteria interactions. Microbiology-SGM **156:**3232-3242.
[33] **Russell, E. G.** 1979. Types and distribution of anaerobic bacteria in the large intestine of pigs. Applied and Environmental Microbiology **37:**187-193.
[34] **Devriese, L. A., L. Laurier, P. Deherdt, and F. Haesebrouck.** 1992. Enterococcal and streptococcal species isolated from feces of calves, Young Cattle and Dairy-Cows. Journal of Applied Bacteriology **72:**29-31.
[35] **Devriese, L. A., J. Hommez, B. Pot, and F. Haesebrouck.** 1994. Identification and composition of the streptococcal and enterococcal flora of tonsils, intestines and feces of pigs. Journal of Applied Bacteriology **77:**31-36.
[36] **Russell, J. B., F. Diez-Gonzalez, and G. N. Jarvis.** 2000. Invited review: Effects of diet shifts on Escherichia coli in cattle. Journal of Dairy Science **83:**863-873.
[37] **Dowd, S. E., T. R. Callaway, R. D. Wolcott, Y. Sun, T. McKeehan, R. G. Hagevoort, and T. S. Edrington.** 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiology **8:**125.
[38] **Oh, P. L., A. K. Benson, D. A. Peterson, P. B. Patil, E. N. Moriyama, S. Roos, and J. Walter.** 2010. Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. Journal of the International Society for Microbial Ecology **4:**377-387.
[39] **Rudi, K., B. Moen, M. Sekelja, T. Frisli, and M. R. F. Lee.** 2012. An eight-year investigation of bovine livestock fecal microbiota. Veterinary Microbiology **160:**369-377.
[40] **Evangelopoulou, G., S. Kritas, A. Govaris, and A. R. Burriel.** 2014. Pork meat as a potential source of *Salmonella enterica subsp arizonae* infection in humans. Journal of Clinical Microbiology **52:**741-744.
[41] **Maskell I, Smithard R.** 1994. Degradation of glucosinolates during *in vitro* incubations of rapeseed meal with myrosinase *(EC* 3.2.3.1) and with pepsin *(EC* 3.4.23.I)-hydrochloric acid, and contents of porcine small intestine and caecum. British Journal of Nutrition **72:**455-466.
[42] **Wu, X.-M., Meijer J**. 1999. *In vitro* degradation of intact glucosinolates by phytophatogenic fungi of Brassica. Proceedings of the 10th International Rapeseed Congress, Canberra, Australia.
[43] **Saavedra, MJ, Dias CSP, Martinez-Murcia A, Bennett RN, Aires A, Rosa EAS.** 2012. Antibacterial effects of glucosinolate-derived hydrolysis products against *Enterobacteriaceae* and *Enterococci* isolated from pig ileum segments. Foodborne Pathogens and Disease. **9:**338-345.
[44] **Le Bizec, B., E. Bichon, Y. Deceuninck, S. Prevost, F. Monteau, J. P. Antignac, and G. Dervilly-Pinel.** 2011. Toward a criterion for suspect thiouracil administration in animal husbandry. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment **28:**840-847.
[45] **Wozniak, B., S. Witek, J. Zmudzki, and A. Klopot.** 2012. Natural Occurrence of thiouracil in urine of livestock in Poland. Bulletin of the Veterinary Institute in Pulawy **56:**611-615.
[46] **Wauters, J., J. Vanden Bussche, B. Le Bizec, J. A. L. Kiebooms, G. Dervilly-Pinel, S. Prevost, B. Wozniak, S. S. Sterk, D. Gronningen, D. Kennedy, S. Russell, P. Delahaut, L. Vanhaecke.** 2014. Towards a new European threshold to discriminate illegally administered from naturally occurring thiouracil in livestock. Journal of Agricultural and Food Chemistry, submitted.
[47] **Wilbur, R. D., D. V. Catron, L. Y. Quinn, V. C. Speer, V. W. Hays.** 1960. Intestinal flora of the pig as influenced by diet and age. Journal of Nutrition. **71:**168-175.
[48] **Kim, H. B., K. Borewicz, B. A. White, R. S. Singer, S. Sreevatsan, Z. J. Tu, and R. E. Isaacson.** 2011. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. Veterinary Microbiology **153:**124-133.
[49] **Esteve-Romero, J., I. Escrig-Tena, E. F. Simo-Alfonso, and G. Ramis-Ramos.** 1999. Determination of thyreostatics in animal feed by micellar electrokinetic chromatography. Analyst **124:**125-128.
[50] **Perez-Ruiz, T., C. Martinez-Lozano, A. Sanz, and R. Galera.** 2005. An ultrasensitive method for the

determination of thiouracil and phenylthiouracil using capillary zone electrophoresis and laser-induced fluorescence detection. Electrophoresis **26:**2384-2390.

[51] **Kong, D. X., Y. W. Chi, L. C. Chen, Y. Q. Dong, L. Zhang, and G. N. Chen.** 2009. Determination of thyreostatics in animal feeds by CE with electrochemical detector. Electrophoresis **30:**3489-3495.

[52] **Vanden Bussche, J., J. A. L. Kiebooms, N. De Clercq, Y. Deceuninck, B. Le Bizec, H. F. De Brabander, and L. Vanhaecke.** 2011. Feed or Food Responsible for the Presence of Low-Level Thiouracil in Urine of Livestock and Humans? Journal of Agricultural and Food Chemistry **59:**5786-5792.

[53] **European Union Reference Laboratories, E. U. R. L.** 2014. EURL reflection paper. Not yet available online.

[54] **Vanden Bussche, J., S. S. Sterk, H. F. De Brabander, M. H. Blokland, Y. Deceuninck, B. Le Bizec, and L. Vanhaecke.** 2012. Thyreostatic drugs, stability in bovine and porcine urine. Analytical and Bioanalytical Chemistry **403:**2973-2982.

[55] **Sjaastad, Ø. V., K. Hove, O. Sand.** 2004. The endocrine system. In: Physiology of the domestic animals, Scandinavian Veterinary Press, Oslo, 2nd ed., pp. 490–563. ISBN: 82-91743-11-8.

[56] **Soldin, O. P., and S. J. Soldin.** 2011. Thyroid hormone testing by tandem mass spectrometry. Clinical Biochemistry **44:**89-94.

[57] **Hegstad-Davies, R. L.** 2006. A review of sample handling considerations for reproductive and thyroid hormone measurement in serum or plasma. Theriogenology **66:**592-598.

[58] **Thienpont, L. M., K. Van Uytfanghe, K. Poppe, and B. Velkeniers.** 2013. Determination of free thyroid hormones. Best Practice & Research Clinical Endocrinology & Metabolism **27:**689-700.

[59] **Lurye, J. C., E. N. Behrend, and R. J. Kemppainen.** 2002. Evaluation of an in-house enzyme-linked immunosorbent assay for quantitative measurement of serum total thyroxine concentration in dogs and cats. Journal of the American Veterinary Medical Association **221:**243-249.

[60] **Kemppainen, R. J., and J. R. Birchfield.** 2006. Measurement of total thyroxine concentration in serum from dogs and cats by use of various methods. American Journal of Veterinary Research **67:**259-265.

[61] **Scigelova, M., M. Hornshaw, A. Giannakopulos, and A. Makarov.** 2011. Fourier Transform Mass Spectrometry. Molecular & Cellular Proteomics **10:** M111.009431–19.

[62] **Herrero, P., N. Cortes-Francisco, F. Borrull, J. Caixach, E. Pocurull, and R. M. Marce.** 2014. Comparison of triple quadrupole mass spectrometry and Orbitrap high-resolution mass spectrometry in ultrahigh performance liquid chromatography for the determination of veterinary drugs in sewage: benefits and drawbacks. Journal of Mass Spectrometry **49:**585-596.

[63] **Brand, W. A.** 1996. High precision isotope ratio monitoring techniques in mass spectrometry. Journal of Mass Spectrometry **31:**225-235.

[64] **Vanhaecke, L., K. Verheyden, J. Vanden Bussche, F. Schoutsen, and H. De Brabander.** 2009. UHPLC Coupled with Fourier Transform Orbitrap for Residue Analysis. Lc Gc Europe **22:**364-374.

[65] **Alvarez-Sanchez, B., F. Priego-Capote, and M. D. L. de Castro.** 2010. Metabolomics analysis II. Preparation of biological samples prior to detection. TrAC-Trends in Analytical Chemistry **29:**120-127.

[66] **Léon, N., M. Roca, C. Igualada, C. P. B. Martins, A. Pastor, and V. Yusa.** 2012. Wide-range screening of banned veterinary drugs in urine by ultra high liquid chromatography coupled to high-resolution mass spectrometry. Journal of Chromatography A **1258:**55-65.

[67] **European Community.** 2002. Commissions Decision 2002/657/EC. Official Journal of the European Community **L 221:** 8-36.

[68] **Sumner, L. W., A. Amberg, D. Barrett, M. H. Beale, R. Beger, C. A. Daykin, T. W. M. Fan, O. Fiehn, R. Goodacre, J. L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A. N. Lane, J. C. Lindon, P. Marriott, A. W. Nicholls, M. D. Reily, J. J. Thaden, and M. R. Viant.** 2007. Proposed minimum reporting standards for chemical analysis. Metabolomics **3:**211-221.

[69] **Pleil, J. D., M. A. Stiegel, and T. H. Risby.** 2013. Clinical breath analysis: discriminating between human endogenous compounds and exogenous (environmental) chemical confounders. Journal of Breath Research **7**:1-11.

[70] **Ellis, C. K., R. S. Stahl, P. Nol, W. R. Waters, M. V. Palmer, J. C. Rhyan, K. C. VerCauteren, M. McCollum, and M. D. Salman.** 2014. A Pilot Study Exploring the Use of Breath Analysis to Differentiate Healthy Cattle from Cattle Experimentally Infected with *Mycobacterium bovis*. Plos One **9:**e89280.

[71] **Mooney, M. H., B. Le Bizec, and C. T. Elliott.** 2009. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. Trac-Trends in Analytical Chemistry **28:**665-675.

[72] **Meier-Augenstein, W.** 1999. Applied gas chromatography coupled to isotope ratio mass spectrometry. J. Chromatogr. A **842:**351-371.

[73] **Le Bizec, B., G. Pinel, and J. P. Antignac.** 2009. Options for veterinary drug analysis using mass spectrometry. J. Chromatogr. A **1216:**8016-8034.

[74] **Scarth, J., C. Akre, L. van Ginkel, B. Le Bizec, H. De Brabander, W. Korth, J. Points, P. Teale, and J. Kay.** 2009. Presence and metabolism of endogenous androgenic-anabolic steroid hormones in meat-producing animals: a review. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment **26:**640-671.

[75] **Kiebooms, J. A. L., J. Vanden Bussche, L. Y. Hemeryck, V. Fievez, and L. Vanhaecke.** 2012. Intestinal Microbiota Contribute to the Endogenous Formation of Thiouracil in Livestock. Journal of Agricultural and Food Chemistry **60:**7769-7776.

[76] **European Union Reference Laboratories.** 2007. EURL Guidance Document. Available online on: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf.
[77] **European Union.** 2009. Commission Regulation No 470/2009. Official Journal of the European Union **L 152:** 11-22.

Summary

At present, thyreostatic drugs, including thiouracil (TU), are still banned from livestock production in the European Union. This banishment is based on the illegal weight gain they cause, as well as, the potential teratogenic and carcinogenic effects their residues might induce. About a decade ago, the occurrence of low-level concentrations (< RC of 10 µg L^{-1}) of TU in livestock urine raised the awareness for a potentially other origin than illicit administration. Over the years, the hypothesis of a feed-related origin (Brassicaceae) was put forward, but the complete elucidation of this natural TU formation remained unfulfilled. Clarification was, however, urgently needed since, sensu stricto, any concentrations of TU in livestock urine were a violation of law, subsequently leading to economical consequences. In this doctoral research, the natural TU formation in livestock was investigated through an in depth multi-disciplinary approach, including analytical developments, static *in vitro* gastrointestinal simulations and microbiological approaches.

In **Chapter I** a general introduction is given concerning the different thyreostatic drugs and their effect on thyroid function. The mechanisms of action of the thyreostatic effect instigated by synthetic and natural occurring goitrogens, as well as, the anatomy and physiology of the thyroid gland, were discussed. Furthermore, the duality induced by the possibility of natural occurrence of thyreostats and their banned status in animal production, required an overview of the current legislative framework surrounding thyreostats. The Brassicaceae, which are believed to lie at the basis of the natural occurrence of thyreostats, were commented on with respect to their application in the animal and human diet. Subsequently, a short preface on LC-MS analysis was given followed by an overview of the analytical evolutions concerning the detection of thyreostats in animal matrices and animal feed, and the detection of thyroid hormones in blood. Finally, the potential of bacterial influence on the occurrence of thyreostats in animal urine evolving from the ingestion of animal feed was discussed. Consequently, an overview of the most common microbiological identification techniques was displayed.
Following the introduction the conceptual framework and the aims of this work were elaborated on.

In **Chapter II** an investigation into the natural TU formation was performed using static *in vitro* digestion (stomach/rumen, small intestine, large intestine) simulations of bovines and porcines with addition of various Brassicaceae representatives (traditional rapeseed, coarse colza '00' meal, broccoli, cauliflower). Thereby aiming to prove the formation of TU was possible in the GI tract and to locate its main

formation site, which turned out to be the large intestine. The influence of several pre-treatments applied to feed (e.g. grounding) and faecal inoculum (e.g. autoclavation) were evaluated to give a first insight into the mechanistic basis of natural TU formation. Furthermore, inter-species and intra-species variations were demonstrated.

In **Chapter III** a more in depth assessment of the influential factors concerning TU formation upon Brassicaceae *in vitro* porcine colonic digestion was performed. Various alterations in the incubation protocol were investigated; including pre-treatments of the inoculum (filter sterilization, pasteurisation, autoclavation, pre-culturing, anaerobicity) and Brassicaceae feed (autoclavation, omission). Based on the results from **Chapters II & III**, the process of natural TU formation could be tentatively elucidated as an anaerobic interaction between Brassicaceae and intestinal microorganisms, producing a membrane-bound extracellular enzyme sustaining moderate heat, but not autoclavation, with glucosinolate degrading activity (myrosinase-like activity).
Moreover, a high TU producing porcine faecal inoculum allowed isolation and identification of common TU producing intestinal bacteria (*Escherichia coli, Enterococcus faecium, Lactobacillus reuteri* and *Salmonella choleraesuis ser. arizonae*), which have been reported in both bovines and porcines. The influence of age, most likely as a result of age-related gastrointestinal microbial changes on TU production was established as well. Finally, it could be concluded that TU formation is directly related to the presence of rapeseed feed and its inherent glucosinolates and/or degradation products thereof, but other sources of TU precursors may not be excluded.

Chapter IV describes the optimization of an extraction procedure for TU from Brassicaceae and non-Brassicaceae feeds based on a fractional factorial Plackett-Burman (PB) design with subsequent validation of the LC-MS^2 detection method. The limit of quantification in animal feeds amounted to 0.5 µg kg^{-1} with both, repeatability and within-laboratory reproducibility (RSD%), below 6%. Satisfying recoveries were attained, ranging between 90.9 and 99.7% for TU. Finally, the applicability of the method was proven on 22 Brassicaceae and non-Brassicaceae feed samples obtained through the Irish national control plan following positive urine monitoring.

In **Chapter V** an UHPLC-MS/MS method was developed and validated for both free and total thyroid hormones (T_3, rT_3 and T_4) in bovine serum. Two separate extraction

procedures were developed by means of Plackett-Burman and D-optimal designs. The limits of quantification for T4, rT3 and T3 were respectively 0.04 ng mL^{-1}, 0.05 ng mL^{-1}, 0.03 ng mL^{-1} for the total fraction, and 6.6 pg mL^{-1}, 2.6 pg mL^{-1} and 2.7 pg mL^{-1} for the free fraction. Individual recoveries of total and free thyroid hormone fractions ranged between 95.6-106.3% and 92.1-106.5%. Good results for repeatability and intra-laboratory reproducibility (RSD%) were observed, i.e. respectively ≤8.0% and ≤7.3% for the total and free fractions. This method will prove its benefits when evaluating the long-term effects of endogenous TU on the thyroid function, in for example *in vivo* studies designed to evaluate the impact of natural thyreostats on thyroid health.

In **Chapter VI** some general conclusions were formulated and future perspectives discussed. Briefly, this study contributed to the general understanding of natural TU detection in livestock urine. More specifically, it offered a potential mechanism for gastrointestinal TU formation upon Brassicaceae feed digestion in livestock. Indeed, several TU forming bacterial strains, which are common intestinal species in livestock (porcine, bovine), were isolated and identified. Based on these results the following future developments are foreseen: the discovery of a biomarker or the development of an analytical method that allows differentiation of the now proven naturally occurring TU in livestock, as well as, an adaptation of the legislative framework, which seems vital. In this context, a thorough evaluation of the long-term health effects of these low-level natural TU residues should be made as well, since man and animal are potentially chronically exposed to those through consumption of products from animal origin and Brassicaceae crops. Besides, the analytical methods developed in this work, could also contribute to the identification of natural thyreostats in livestock, through evaluation of the effect of natural thyreostats on the thyroid function *in vivo* or by assessing TU in animal feeds to correlate possible findings with suspected low-level TU positive urine samples, as prescribed by the 2014 EURL Reflection paper. Moreover, the thyroid hormone UHPLC-MS/MS method could after further optimization easily lend itself to other species (e.g. dogs, cats and horses), which would mean a great improvement in current veterinary medicine, since MS methods have not yet been implemented, as opposed to human medicine.

Samenvatting

Tot op heden is het gebruik van thyreostatica, zoals thiouracil (TU), tijdens de dierlijke vetmesting verboden in de Europese Unie. Dit omwille van de illegale gewichtstoename die ze veroorzaken, evenals door de teratogene en carcinogene effecten die hun residuen met zich mee kunnen brengen.
Het laatste decennium werden sporadisch lage TU-concentraties (<RC van 10 µg L^{-1}) gedetecteerd in de urine van voedselproducerend vee, waardoor het idee ontstond dat er mogelijks een andere oorzaak dan illegale toediening aan de basis zou kunnen liggen van de detectie van lage TU-concentraties. In de loop der jaren werd de hypothese van een voedingsgerelateerde oorsprong (Brassicaceae) naar voren geschoven, maar de volledige opheldering van deze natuurlijke TU-vorming bleef onvervuld. Aangezien sensu stricto, iedere TU-concentratie in urine een overtreding van de wet vormt en vervolgens kan leiden tot economische sancties, is de ontrafeling van deze problematiek wel hoogst noodzakelijk. Daarom werd in dit doctoraatsonderzoek het ontstaansproces van natuurlijke TU-vorming in voedselproducerende dieren onderzocht door een diepgaande multidisciplinaire aanpak, wat zowel de ontwikkeling van analytische methoden, de toepassing van microbiologische benaderingen en statische *in vitro* gastro-intestinale simulaties omvatte.

In **Hoofdstuk I** wordt een algemene inleiding gegeven over de verschillende thyreostatica en hun effect op de schildklierfunctie. De mogelijke werkingsmechanismen van het schildklierremmend effect veroorzaakt door synthetische en natuurlijk voorkomende goitrogenen wordt belicht, alsmede de anatomie en fysiologie van de schildklier. Bovendien vereist de dualiteit die ontstond door het mogelijks natuurlijk voorkomen van thyreostatica en hun verboden statuut binnen de dierlijke voedselproductie, een overzicht van het huidig wettelijk kader omtrent thyreostatica. De Brassicaceaegewassen, die vermoedelijk aan de basis liggen van de natuurlijke aanwezigheid van thyreostatica in vee, worden eveneens besproken met betrekking tot hun consumptie door mens en dier. Vervolgens wordt een korte algemene inleiding over LC-MS-analyse gegeven, gevolgd door een overzicht van de evolutie van de analytische technieken enerzijds, voor de opsporing van thyreostatica in dierlijke matrices en diervoeder, en anderzijds, voor de detectie van schildklierhormonen in het bloed. Tot slot wordt besproken hoe de detectie van lage thyreostaticaconcentraties in dierlijke urine zou beïnvloed kunnen worden door de activiteit van bacteriën op Brassicaceaediervoeders, wat gevolgd wordt door

een noodzakelijk overzicht van de meest voorkomende microbiologische identificatietechnieken.
Na de introductie werden het conceptueel kader en de doelstellingen van dit werk uiteengezet.

In **Hoofdstuk II** wordt het onderzoek naar de natuurlijke TU-vorming in varkens en runderen, dat met behulp van statische *in vitro* digestie simulaties (maag / pens, dunne darm, dikke darm) met toevoeging van diverse Brassicaceaevariëteiten (traditioneel koolzaad, koolzaad '00' schroot, broccoli en bloemkool) werd uitgevoerd, beschreven. Hieruit bleek dat de vorming van TU *in vitro* kon plaatsvinden in het maagdarmkanaal, en dat de belangrijkste vorming zich situeerde ter hoogte van de dikke darm. De invloeden van verschillende voorbehandelingen van diervoeders (bv. pletten) en fecaal inoculum (bv. autoclaveren) werden geëvalueerd om een eerste interpretatie aangaande de mechanistische basis van de natuurlijke TU-vorming te kunnen vormen. Bovendien werden inter- en intraspeciesvariaties met betrekking tot TU-vorming aangetoond.

In **Hoofdstuk III** werd een diepgaandere evaluatie van de factoren die de vorming van TU beïnvloeden, uitgevoerd op basis van *in vitro* Brassicaceae varkenscolonsimulaties. Verschillende variaties op het incubatieprotocol werden onderzocht, waaronder voorbehandelingen van het fecaal inoculum (bijvoorbeeld filtersterilisatie, pasteurisatie, autoclavering, opkweken, anaerobiciteit) en het Brassicaceaevoeder (autoclavering, weglaten ervan). Gebaseerd op de teruggevonden resultaten (**Hoofdstuk II en III**) werd het proces van natuurlijke TU-vorming voorlopig gekarakteriseerd als een anaerobe interactie tussen Brassicaceae en intestinale micro-organismen. Deze laatste zouden op hun beurt een membraangebonden extracellulair enzyme produceren dat bestand is tegen matige hitte, maar niet tegen autoclavering en dat een glucosinolaatafbrekende werking (myrosinase-achtige activiteit) bezit.
Daarnaast werd de isolatie en identificatie van TU-producerende bacteriële stammen (*Escherichia coli, Enterococcus faecium, Lactobacillus reuteri* en *Salmonella enterica ssp. arizonae*), die zowel bij runderen als bij varkens reeds gerapporteerd werden, uitgevoerd met behulp van een sterk TU-producerend fecaal inoculum (varken). Tevens kon vastgesteld worden dat leeftijd een rol speelt in de intestinale TU-vorming, dit waarschijnlijk als gevolg van leeftijdsgerelateerde microbiële veranderingen. TU-vorming bleek ook rechtstreeks verband te houden met de aanwezigheid van koolzaadvoeders en de aanwezige glucosinolaten en/of

afbraakproducten daarvan, maar daarnaast mogen ook andere bronnen van TU-precursoren niet uitgesloten worden.

Hoofdstuk IV beschrijft de optimalisatie van een extractieprocedure voor TU uit Brassicaceae- en niet-Brassicaceaevoeders op basis van een fractioneel factorieel Plackett-Burmanmodel (PB) met de daaropvolgende validatie van de LC-MS^2-detectiemethode. De bepalingsgrens in diervoeders bedroeg 0.5 µg kg^{-1} met zowel voor herhaalbaarheid als voor de intralaboratorium reproduceerbaarheid (RSD%) waarden onder de 6.0%. Een toereikende terugvinding, variërend tussen 90.9 en 99.7%, werd bereikt voor TU. Uiteindelijk werd de toepasbaarheid van de methode aangetoond op 22 Brassicaceae- en niet-Brassicaceaevoedermonsters afkomstig van het Ierse nationale controleplan, die verkregen werden na opvolging van TU-positieve urinestalen.

In **Hoofdstuk V** werd een UHPLC-MS/MS methode ontwikkeld en gevalideerd voor zowel vrije als totale schildklierhormonen (T_3, rT_3 en T_4) in bovien serum. Twee afzonderlijke extractieprocedures werden ontwikkeld door middel van PB en D-optimale modellen. De respectievelijke kwantificatiegrenzen voor T_4, rT_3 en T_3 bedroegen 0.04 ng mL^{-1}, 0.05 ng mL^{-1}, 0.03 ng mL^{-1} voor de totale fractie, en 6.6 pg mL^{-1}, 2.6 pg mL^{-1} en 2.7 pg mL^{-1} voor de vrije fractie. Individuele terugvinding van totaal en vrij schildklierhormoonfracties varieerde tussen 95.6-106.3% en 92.1-106.5%. Goede resultaten voor de herhaalbaarheid en de intralaboratorium reproduceerbaarheid (RSD%) werden waargenomen, respectievelijk ≤8.0% en ≤7.3% voor de totale en vrije fracties. Deze methode zal zijn voordeel bewijzen bij het evalueren van de langetermijneffecten van endogeen TU op de schildklierfunctie. Dit kan bijvoorbeeld in *in vivo* studies, die de impact van natuurlijke thyreostatica op de schildklierwerking wensen te evalueren.

In **Hoofdstuk VI** worden enkele algemene conclusies geformuleerd en toekomstperspectieven besproken. Kortom, heeft deze studie bijgedragen tot een beter inzicht betreffende de detectie van natuurlijk TU in dierlijke urine door het verlenen van een mechanistische uitleg voor de gastrointestinale TU-vorming door Brassicaceaevertering in vee. Sterker nog, een aantal TU-vormende bacteriestammen, die gemeenschappelijke intestinale soorten zijn in vee (varkens, runderen), werden geïsoleerd en geïdentificeerd. Op basis van deze resultaten werden toekomstperspectieven geformuleerd, die o.a. het zoeken naar een biomerker voor differentiatie van het inmiddels bewezen natuurlijk voorkomen van TU

in vee en de aanpassing van het wettelijk kader, beoogden. In deze context zou eveneens een grondige evaluatie van de langetermijneffecten van deze lage natuurlijke TU-residuen op de gezondheid van mens en dier moeten gemaakt worden, aangezien een mogelijke chronische blootstelling door consumptie van producten van dierlijke oorsprong en Brassicaceaegewassen niet kan uitgesloten worden. Daarnaast zullen de analytische methoden die in dit werk ontwikkeld werden eveneens kunnen bijdragen aan het verdere onderzoek naar natuurlijke thyreostatica in vee. Dit, enerzijds, in het kader van een *in vivo* evaluatie van het effect van natuurlijke thyreostatica op de schildklierfunctie en, anderzijds, door de beoordeling van TU in diervoeders zodat de resultaten teruggekoppeld zouden kunnen worden aan TU-positieve urinemonsters, zoals voorgeschreven door de 2014 EURL discussienota. Bovendien zou de ontwikkelde UHPLC-MS/MS detectiemethode voor de analyse van schildklierhormonen in bovien serum zich na enige verdere optimalisatie eenvoudig kunnen lenen tot toepassing voor andere diersoorten (zoals honden, katten en paarden). Dit zou alvast een grote verbetering betekenen in de hedendaagse diergeneeskunde, aangezien MS-methoden daar nog niet toegepast worden, dit in tegenstelling tot de humane geneeskunde.

Curriculum vitae

Personalia

Name:	Kiebooms Jullie Anna Lucie
Email:	kiebooms.j@gmail.com
City of recidence:	Antwerp, Belgium
Date of birth:	20th of August 1984
Nationality:	Belgian

Education

2002 **Latin-Modern languages**

Onze-Lieve-Vrouwecollege, Antwerp

2010 **Master in Veterinary Sciences, Large Animals training program (Equine), with a senior year specialization in 'Research and Industry'**

Ghent University, Merelbeke, Belgium

Thesis: *Micro-pollutants in the coastal ecosystem: Development of an analytical method for determination of pharmaceuticals in biota from the Belgian coastal zone.*

2014 **Phd-thesis** (started in 2010)

Department of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis, Ghent University, Merelbeke, Belgium.

Professional activities

Oct. 2010	School for Advanced Residue Analysis in Food (2 weeks) organised by the Laboratory for residues and contaminants in food (LABERCA) at Nantes Atlantic College of Veterinary Medicine, Food Science and Engineering (ONIRIS), Nantes, France.
2010-2014	*Mentor* of 4 master students from the Faculty of Veterinary Medicine in the fulfilment of their *literature study*.
2010-2013	*Mentor* of 3 master students from the Faculty of Veterinary Medicine or Pharmaceutical sciences in the fulfilment of their *research thesis.*
Apr. 2011	Communication group: Trace element mycotoxines and phycotoxines, pesticides, and hormones - veterinary drugs (96/23). Organized by the Scientific Institute for Public Health (WIV) and The Veterinary and Agrochemical Research Centre (CODA-CERVA), Brussels, Belgium.
Oct. 2012	PhD-course: Introduction to SPSS, by , Institute for Continuing Education in Science (IPVW-ICES) Ghent, Belgium.
Oct. 2012	PhD-course: Creative thinking, by K. Raats, Ghent, Belgium.
Dec. 2012	PhD-course: Advanced academic English: effective slide design, University Language Centre (UCT), Ghent, Belgium.
2012-2013	*Tutor-assistant* for the practical sessions of the course 'Food and environmental chemistry' (2nd Bachelor students) and the course 'Applied

	biomedical techniques' (3rd year Master students) at the Faculty of Veterinary Medicine Merelbeke, Belgium.
Oct. 2013	Update Laboratory Animal course: 'Changes in the legislation on the protection of experimental animals' by Prof. K. Hermans (Ethical committee president), Faculty of Veterinary Medicine, Merelbeke, Belgium.
Mar. 2014	PhD-course: 'Proteomics: Mass spectrometry data handling' by Flemish Institute of Biotechnology-Bioinformatics training and services (VIB-BITS), Ghent, Belgium.
Apr. 2014	PhD-course: 'Basics of biology for engineers' at Imec Academy, University of Leuven, Belgium.
May 2014	Laboratory Animal course: 'Species-specific welfare in the laboratory: rats and mice' by Dr. H. Blom at the Faculty of Veterinary Medicine, Merelbeke, Belgium.
Sept-Dec. 2014	*Research assistant* on the THYREOMERK FOD-project (RF12/6260) at the Laboratory of Chemical Analysis, Faculty of Veterinary Medicine, Merelbeke, Belgium.

PUBLICATIONS

Wille, K., **J. A. L. Kiebooms**, M. Claessens, K. Rappé, J. Vanden Bussche, N. Van Praet, E. De Wulf, P. Van Caeter, R. C. Janssen, H. F. De Brabander, L. Vanhaecke. 2011. Development of analytical strategies using U-HPLC-MS/MS and LC-ToF-MS for the quantification of micropollutants in marine organisms. Analytical and Bioanalytical Chemistry **400:**1459–1472.

Vanden Bussche, J., **J. A. L. Kiebooms**, N. De Clercq, K. Wille, K. Bekaert, Y. Deceuninck, B. Le Bizec, H. F. De Brabander, L. Vanhaecke. 2011. Feed or food responsible for the presence of low-level thiouracil in urine of livestock and humans? Journal of Agricultural and Food Chemistry **59:**5786-5792.

Kiebooms, J. A. L., J. Vanden Bussche, L. Y. Hemeryck, V. Fievez, L. Vanhaecke. 2012. Intestinal microbiota contribute to the endogenous formation of thiouracil in livestock. Journal of Agricultural and Food Chemistry, Journal of Agricultural and Food Chemistry, **60:**7769–7776.

Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche, L. Vanhaecke. 2014. Validated ultra high performance liquid chromatography-tandem mass spectrometry method for quantitative analysis of total and free thyroid hormones in bovine serum. Journal of Chromatography A **1345:**164–173.

Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche, K. Houf, P. De Vos, S. Van Trappen, I. Cleenwerck and L. Vanhaecke. 2014. Identification and characterisation of thiouracil forming bacteria upon porcine *in vitro* digestion of Brassicaceae feed. Applied and Environmental Microbiology, **80(23):**7433-7442.

Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche, D. Healey and L. Vanhaecke. 2015. Validation of a quantitative method using liquid chromatography coupled to multiple mass spectrometry for thiouracil in feedstuffs used in animal husbandry. Analytical and Bioanalytical Chemistry, *in press* (DOI: 10.1007/s00216-014-8347-x).

Wauters, J., J. Vanden Bussche, B. Le Bizec, **J. A. L. Kiebooms**, G. Dervilly-Pinel, S. Prevost, B. Wozniak, S. S. Sterk, D. Gronningen, D. Kennedy, S. Russell, P. Delahaut, and L. Vanhaecke. 2014. Towards a new European threshold to discriminate illegally administered from naturally occurring thiouracil in livestock. Journal of Agricultural and Food Chemistry, *submitted.*

Without peer review

Vanden Bussche, J., **J. A. L. Kiebooms**, N. De Clercq, K. Wille, K. Bekaert, Y. Deceuninck, B. Le Bizec, H. F. De Brabander, L. Vanhaecke. 2011. First evidence of a natural origin for banned drug that plumps up livestock. Press release for the American Chemical Society (ACS) Office of Public Affairs' Weekly PressPac.

Abstracts

Kiebooms, J. A. L., J. Vanden Bussche, H. F. De Brabander and L. Vanhaecke. 2011. Brassicaceae derived food and feed responsible for low level thiouracil residues. Koninklijke Vlaamse Chemische Vereniging (KVCV) Symposium: Mass Spectrometry in Food and Feed, Merelbeke, Belgium.

Kiebooms, J. A. L., J. Vanden Bussche, H. F. De Brabander and L. Vanhaecke. 2011. Brassicaceae derived food and feed responsible for low level thiouracil residues. EuroFoodChem XVI: Translating food chemistry into health benefits, Gdanks, Poland. Polish Journal of Food and Nutrition Sciences. **61(suppl. 1):**21-P8.

Kiebooms, J. A. L., J. Vanden Bussche, H. F. De Brabander and L. Vanhaecke. 2012. Does gastrointestinal digestion affect low level thiouracil residues upon Brassicaceae derived food and feed consumption? Residues of Veterinary Drugs in Food: proceedings of the EuroResidue VII conference, Egmond-aan-Zee, Netherlands.

Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke. 2013. Plackett-Burman design for the extraction of thyreostats with LC-MS^2 detection in Brassicaceae feeds. Recent Advances in Food Analysis (RAFA), 6th International symposium, Prague, Czech Republic.

Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke. 2013. Plackett-Burman design for the extraction of thyreostats with LC-MS^2 detection in Brassicaceae foods and feeds. KVCV Symposium: Trends in Food Analysis VII, Ghent, Belgium

Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke. 2014 Thyroid hormones in livestock serum: extraction optimization by fractional factorial design followed by UHPLC-MS/MS detection. 13th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-13), Bruges, Belgium.

Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke. 2014. Optimization of thyreostat analysis from animal feeds by Plackett-Burman and LC-MS^2. Seventh International Symposium on Hormone and Veterinary Drug Residue Analysis, Ghent, Belgium.

Kiebooms, J. A. L., J. Vanden Bussche and L. Vanhaecke. 2014. Isolation and identification of thiouracil forming bacteria from livestock feces. Seventh International Symposium on Hormone and Veterinary Drug Residue Analysis, Ghent, Belgium.

Conferences, workshops and seminars

- 'HRMS mass spectrometry: Orbitrap operations training course' by Thermo Scientific, September 13th -14th, 2010, Merelbeke, Belgium.

- Belgian Association for Meat Science and Technology (BAMST) Symposium: Energie- en zoutreductie in vlees en vleesproducten - October 21th, 2010, Merelbeke, Belgium.

- Seminar on latest technologies in sample preperation and solid phase extraction, by Dionex Benelux, February 2011, Antwerp, Belgium.

- KVCV Symposium: Mass Spectrometry in Food and Feed **(poster presentation)**: June 9th, 2011, Merelbeke, Belgium.
  Title: "Brassicaceae derived food and feed responsible for low level thiouracil residues" - Kiebooms, J. A. L., J. Vanden Bussche, H. F. De Brabander and L. Vanhaecke.

- Euro Food Chem XVI Congress: Translating food chemistry in health benefits **(poster presentation)**: July 5th-8th, 2011, Gdansk, Poland.
  Title: "Brassicaceae derived food and feed responsible for low level thiouracil residues" - Kiebooms, J. A. L., J. Vanden Bussche, H. F. De Brabander and L. Vanhaecke.

- EuroResidue VII Conference (**oral presentation**): May 14th-16th 2012, Egmond-aan-Zee, The Netherlands.

Title: "Does gastrointestinal digestion affect low level thiouracil residues upon Brassicaceae derived food and feed consumption?" - Kiebooms, J. A. L., J. Vanden Bussche, H. F. De Brabander and L. Vanhaecke.

- 13th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-13) **(poster presentation)**: January 28th-31st 2013, Bruges, Belgium.
  Title: "Thyroid hormones in livestock serum: extraction optimization by fractional factorial design followed by UHPLC-MS/MS detection" – Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke.

- Koninklijke Vlaamse Chemische Vereniging (KVCV) Symposium: Trends in Food Analysis VII **(poster presentation)**: Septemeber 19th 2013, Ghent, Belgium.
  Title: "Plackett-Burman design for the extraction of thyreostats with LC-MS^2 detection in Brassicaceae foods and feeds" - Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke.

- Recent Advances in Food Analysis (RAFA), 6th International symposium **(poster presentation)**: November 5th-8th 2013, Prague, Czech Republic.
  Title; "Plackett-Burman design for the extraction of thyreostats with LC-MS^2 detection in Brassicaceae feeds" - Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke.

- Seventh International Symposium on Hormone and Veterinary Drug Residue Analysis (**oral presentation**): June 2nd -5th 2014, Ghent, Belgium.
  Title: **"**Isolation and identification of thiouracil forming bacteria from livestock feces" - Kiebooms, J. A. L., J. Vanden Bussche and L. Vanhaecke.

- Seventh International Symposium on Hormone and Veterinary Drug Residue Analysis (**poster presentation**): June 2nd -5th 2014, Ghent, Belgium.
  Title: **"**Optimization of thyreostat analysis from animal feeds by Plackett-Burman and LC-MS^2" - Kiebooms, J. A. L., J. Wauters, J. Vanden Bussche and L. Vanhaecke.

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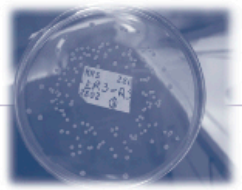
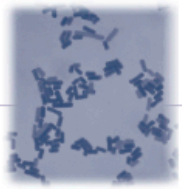
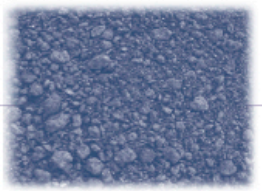
"Poppeke gezien, kasken dicht"

Julie

1 december 2014

Endogenous formation of thiouracil in livestock: mechanistic and analytical aspects

Julie A. L. Kiebooms

2014